# NEUTROPHILS SENSE MICROBE SIZE AND SELECTIVELY RELEASE NEUTROPHIL EXTRACELLULAR TRAPS TO CONTROL LARGE PATHOGENS

---

HANNI NORA BRANZK

Molecular Immunology
The Francis Crick Institute - Mill Hill Laboratory
The Ridgeway, London NW7 1AA

Supervised by Dr Venizelos Papayannopoulos

Submitted to
University College London
for the degree of Doctor of Philosophy

April 2016

Examination board:

Prof Sussan Nourshargh
Prof Caetano Reis e Sousa

Thesis committee:

Prof Brigitta Stockinger
Dr Andreas Wack
Dr Pavel Tolar

I, Hanni Nora Branzk confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Neutrophils are innate immune phagocytes that are critical for antimicrobial defence. Neutrophils are thought to control infection by implementing a single antimicrobial program. It is unclear whether they are able to adapt their antimicrobial strategies to the different types of microbes they encounter. Neutrophil extracellular traps (NETs) are web-like structures that are composed of decondensed chromatin, decorated with neutrophil antimicrobial proteins. NETs have been shown to trap and kill a variety of microbes including parasites, fungi, bacteria and even viruses but their relevance for immune defence *in vivo* remained unresolved. Furthermore, it was unknown whether neutrophils regulate NET release to selectively targeted specific microbes.

This work examined the role of NETs in antimicrobial defence. Specifically, we asked whether NET release is a regulated process that targets distinct sets of microbes and how regulation of NETosis was achieved. Furthermore, we examined the relevance of NETs against different microbes *in vivo*. Finally, we investigated which receptors were involved in triggering NET release in response to the fungus *Candida albicans.*

Using dimorphic fungi and bacteria we found that the neutrophils regulate their antimicrobial strategies depending on the size of the microbe they encounter. Small microbes such as *C. albicans* yeast are phagocytosed, whereas large microbes such as *C. albicans* hyphae trigger NET formation. NETs were critical for the clearance of large hyphae but were dispensable against small yeast in mice. These data explain the specific susceptibility to fungal infection observed in MPO-deficient patients, whose neutrophils are defective in NET formation.

Importantly, we found that phagocytosis is a negative regulator of the NETosis pathway. Sequestration of neutrophil elastase in the phagosome inhibits its translocation to the nucleus, a crucial requirement for NETosis. This strict regulation was crucial since excessive release of NET caused tissue damage and immune pathology.

Furthermore, we identified a set of *C. albicans* cell wall mutants that failed to trigger NETosis, indicating that N- and O-linked mannosylation of cell wall proteins might be implicated in NET induction. Consistently, preliminary results suggest the involvement of TLR4 and MR in the initiation of the NETosis pathway upon stimulation with *C. albicans.*

**This thesis would not have been possible without you..**

First of all I want to thank Veni (aka "the boss"): Being part of your lab was one of the best things that could have happened to me scientifically. I am immensely grateful for all the opportunities I was given by you and for the trust you had in me from the first day. I hadn't even unpacked my suitcase when we were already writing a review, I wasn't even done with my first year when you gave me a student to supervise and it wasn't long after that that you told me to start putting together a manuscript for my paper. This confidence you have in me, is what pushes me to do my best all the time. Thank you for making me an equal partner in our numerous scientific discussions and giving me the freedom to speak my mind. The excitement that I feel when we cover the whiteboard with new (exciting!) ideas is when I remember why I love science. I learned so much and developed as a scientist in these 3 ½ years. Thank you for having me in the lab.

If there were something like a second authorship in a thesis, it would belong to you Michael. Without you none of this would have been possible. You are my pillar of support, you are my best friend, the partner of my dreams, and.. because it's too good to be true.. you are also an amazing scientist. When we talk about my projects, you challenge my views and ask critical questions, you give me input and practical help. You understand how it feels to be in the lab for 10 hours or more every day and you know the pressure and worries that come with it at times. But you also understand and share the joy of having a phenotype or getting a Western to work. Thank you for your endless support and making it work over the distance. You are the best thing that ever happened to me and I am the happiest puppy knowing that I will finally come home and spend the rest of my life with you. Jeg elsker dig så meget.

Kori! I will miss you so much. Thank you for everything you did for me. You are really the best friend and colleague I could have asked for. Whenever I know you are not in the lab, I don't feel like going to work. You are smart and kind and funny. Thank you for stepping out of your way to make this lab a

good place. Thank you for being my shoulder to cry on, for making me hazelnut coffee and listening to my silly stories. Thank you for being my godmother and so much more.

Qian, thank you for all your help every single day. Thank you for your company in the morning before everyone else comes to the lab and for being the warm-hearted person you are. I will miss you.

Thank you Ola and Dorita for being the best students to work with. You made being a supervisor feel like a piece of cake. Ola, thank you so much for your help with the paper. I am happy to see you thrive in your own PhD project now; I always knew you would go your way. Keep on being awesome! Poulakimou, thank you for making the transition so easy. I have a very good feeling leaving this project in your hands, seeing that you already begin to make it your own. I know you will do great in your PhD and I am already looking forward to see you in a few years time.

Thanks to all the other wonderful people that came through the lab. Especially Ryan and Hanna, it was a pleasure to work with you.

Thank you Stefania and Andreas for being good friends and my substitute parents here in London. Andreas, thank you for being my source of non-scientific literature. I know you like to pretend that I didn't like any of the books you gave me anyway, but we both know that this is not true.

I would like to thank the members of my thesis committee Gitta Stockinger, Andreas Wack and Pavel Tolar for the interesting discussions and the valuable advice on this thesis.

Thank you to all the people that work the wonders in this institute. Ade and the building C crew for making sure our mice (and we) are happy. Radma and Radhi for your support with the histology part of this PhD. A particular thanks goes to Donald, who is some sort of C-3PO human-machine interpreter and from whom I learned most of what I know about

microscopes. Thank you to the colleagues who donated blood for this project. And finally all the other great scientists up and down the corridors who make the NIMR such a special place to be.

Thanks to Valeria, Amy and Manpreet. You may not know, but you really made a difference to me.

I would like to thank Dr Lena Alexopoulou and Prof Marina Freudenberg for paving the way to this PhD. It is thanks to your generosity and patience that I continued to pursue this career. You took me in in your laboratories when I was just an undergraduate or master student and entrusted me with projects that nourished my interest in science. It is your investment in this young person I was, that I carry in my heart as one of the greatest fortunes.

Thank you to all the amazing people that stuck with me throughout this journey. Joao, for your friendship that is lasting over the big pond. You have been a friend when I needed it most. Stephe, for showing me that it's never too late to pick up the pieces and reinvent yourself. Anja, for being one of the kindest people I have ever met. Your amazing letters really made me go through the last stretch of this thesis. I admire you for your strength and optimism. You are a great person to be around. Susi, for being a constant inspiration not to take anything too seriously and for putting into perspective the "struggles" that a PhD really are. Natty, without whom I wouldn't have survived uni. I feel blessed that you are still part of my live.

Thank you to my family, who still loves me even though I have horribly neglected them in favour of this PhD. I am sorry I didn't come to your birthdays, anniversaries, baptisms, confirmations and other important events in your lives. Know that your support means the world to me. I want to especially thank my parents Marion and Maik. You are an endless inspiration to me. It is thanks to you that I am constantly trying to be the best version of myself. Thank you for believing in me and supporting me in every decision I make.

# *1 INTRODUCTION*

This study addresses whether neutrophils are able to regulate their antimicrobial strategies. Specifically, we investigated whether neutrophil extracellular traps (NETs) are employed selectively. Since neutrophils are crucially involved in antifungal immunity, we employed the dimorphic fungus *Candida albicans* as a model for the variability of microbes. Therefore, the following chapters will introduce what was known about neutrophil immunity and the release of NETs specifically in the context of antifungal immunity.

## *The immune system*

Immunity is an organism's defence against infection. It requires the capacity to distinguish infectious particles from the host's own tissues. Some form of immune defence is present in nearly all forms of life, including plants and unicellular organisms. Bacteria use enzymes to defend themselves against bacteriophage infection. Immune systems gained complexity during evolution. Whereas innate immunity is found in all plants and animals, adaptive immunity occurred first in jawed vertebrates (Flajnik and Kasahara, 2010).

In higher organisms the immune system has a layered structure. As the first line of defence, skin and mucosal lining provide mechanical, biological and chemical barriers. Thereafter, innate leukocytes (white blood cells) are immediately available. The innate immune system is not specific against any particular pathogen but combats a wide range of microbes. Broad pathogen associated molecular patterns (PAMPs) are recognised by pre-formed pattern recognition receptors (PRRs). In higher vertebrates, innate and adaptive immunity work together. If the innate immune system is not sufficient to resolve an infection, an adaptive immune response is triggered. Adaptive immunity is highly specific and adapts to infection with distinct microbes through production of unique receptors. Adaptive immunity takes time to develop and is continuously reshaped throughout the lifetime of an organism. It confers immunological memory, allowing for a rapid recall

response upon re-infection with the same microbe. Protective vaccination is based on immunological memory (Murphy, 2012).

Immune cells are produced mainly in the bone marrow (BM) where they develop and mature. A common hematopoietic stem cell gives rise to most of the many different hematopoietic lineages.

Innate immune cells include macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, basophils, and natural killer (NK) cells. These cells have direct antimicrobial effects by release of toxic factors or elimination of small microbes via phagocytosis. Innate immune cells are also important activators of the adaptive immune system via the release of signalling molecules called cytokines. The complement system forms another important part of innate immunity. Different complement proteins coat microbial surfaces and mark them for elimination (Murphy, 2012).

The adaptive immune system depends on B lymphocytes and T lymphocytes, which represent the humoral and the cell-mediated immune response, respectively. Both, B and T cells carry highly specific receptors that recognise only a single antigen. These antigen-specific receptors are produced through a process called somatic recombination in the early stages of B and T cell maturation. T cells carry their receptors (TCR) on their surface and recognise antigens presented by the major histocompatibility (MHC) complex. The main classes of T cells are killer T cells, T helper cells and regulatory T cells. Dependent on their phenotype, T cells either directly kill infected or dysfunctional cells or instruct other immune cells to do so. When B cells recognise antigen through their receptor (BCR), they internalise it and present it on their surface. Subsequent activation of these B cells leads to formation of plasma cells, which then secrete millions of copies of their specific receptor in the form of antibodies. Antibodies circulate in the blood, where they mark (opsonise) pathogens that carry the specific antigen. Opsonisation leads to complement activation or uptake by phagocytes (Murphy, 2012).

Activation of T and B cells leads to formation of antigen-specific memory cells. In the event of re-infection with the same pathogen, memory cells can mount a faster and stronger response, mediating protection. Memory was long thought to be a unique feature of the adaptive immune system. However, the recent discovery of trained innate immunity, a form of innate memory, challenges this dogma (van der Meer, 2015).

## *1.1 Neutrophils*

Neutrophils are the most abundant immune cells in the human body and neutrophil deficiencies lead to severe infections, which are often opportunistic. Neutrophils are characterised by a lobulated nucleus and are therefore characterised as polymorphonuclear cells (PMNs). They carry different pre-formed types of granules that contain more then 700 different antimicrobial granule proteins (Borregaard, 2007). Neutrophils develop in the bone marrow (BM) and are terminally differentiated in the blood. The neutrophil life span varies with species and ranges from several hours to days (Pillay, 2010; Tak, 2013). It is markedly increased upon activation at inflammatory sites. Neutrophils are recruited to tissues in response to microbial molecules and cytokines produced by tissue resident cells. These include IL-1β, IL-6, TNF-α, IL-8 as well as IL-17. Neutrophils arrive in great numbers as the first cells at the site of infection. They extravasate through the endothelium after endothelial signals and rolling on the endothelial walls (Kolaczkowska and Kubes, 2013; Nourshargh and Alon, 2014). In the tissues neutrophils are fully equipped for a range of different antimicrobial strategies including degranulation, phagocytosis, release of neutrophil extracellular traps, autophagy, apoptosis and pyroptosis (Kruger, 2015). Furthermore, neutrophils activate other immune cells such as DCs, macrophages, NK cells, B cells and T cells (Mantovani, 2011). However, neutrophils are also implicated in a range of diseases including cancer, metabolic and inflammatory diseases (Bardoel, 2014).

### 1.1.1 Neutrophil production and release

#### *Granulopoiesis*

Approximately 1-2x$10^{11}$ neutrophils are produced daily in humans, making granulopoiesis the greatest activity of the BM. The hematopoietic stem cells (SCs) are localised in osteoblast niches that are characterised by low blood flow and low oxygen levels. More mature cells migrate out of the BM

sinusoids and are based on the abluminal site of the vasculature (Winkler, 2010).

Granulocyte-colony stimulating factor (G-CSF) is important throughout all stages of neutrophil development, including production, proliferation, survival, differentiation and mobilisation into tissues (Semerad, 2002). G-CSF is produced in different tissues upon stimulation with inflammatory mediators such as IFN-β, TNF-α, IL-1 or IL-17. Endothelial cells and macrophages are a prominent source of G-CSF (Roberts, 2005). Despite the importance of G-CSF in neutrophil development, G-CSF-deficient mice still support 25% granulopoiesis and can produce mature neutrophils (Lieschke, 1994). Conventional DCs control the production of G-CSF. Depletion of DCs leads to accumulation of G-CSF and loss of neutrophils from the bone marrow followed by secondary neutrophilia (Jiao, 2014).

Granulopoiesis starts with the development of precursors in the stem cell pool. Thereafter, terminal differentiation is comprised of a mitotic pool and a post-mitotic pool that are separated by cell cycle exit (Klausen, 2004). Post-mitotic BM neutrophils constitute 95% of the neutrophils in the body and rapidly enter circulation.

The self-renewable hematopoietic stem cells (HSC) are present in the BM in low numbers where they are maintained in the BM niches by interaction with stromal cells such as osteoblasts (Orkin and Zon, 2008). HSC lose their self-renewing capacity when they turn into multipotent precursors (MPP) (Görgens, 2013). Under the influence of the transcription factor GATA-1 MPP develop into erythromyeloid progenitors (EM) that give rise to the other granulocyte members eosinophils and basophils (**Figure I-1**). For myeloid lineage commitment, activation of the transcription factor PU.1 is required (Iwasaki, 2005; Nerlov and Graf, 1998) leading to development of lymphomyeloid progenitors (LMP) (Arinobu, 2007; McKercher, 1996). The last step of precursor development is the subsequent formation of granulocyte/monocyte precursors (GMP). Continued PU.1 activity drives

monocyte commitment. GMP that are under the influence of CCAAT/enhancer binding protein α (C/EBPα), continue towards terminal granulocytopoiesis leading to formation of mature granulocytes. C/EBPα regulates the expression of proteins that are necessary for neutrophil differentiation, such as the G-CSF receptor (Radomska, 1998). The NF-κB subunit p50 activates C/EBPα alone or in complex with C/EBPα, to induce enhanced G-CSF production. The first stage of C/EBPα-dependent terminal differentiation is the formation of myeloblasts (Dahl, 2003; Radomska, 1998; Reddy, 2002), which are comparatively small and do not contain granule proteins. Like C/EBPα, the transcription factor growth factor independent-1 (Gfi-1, also Lef-1) is activated in the early stages of terminal differentiation

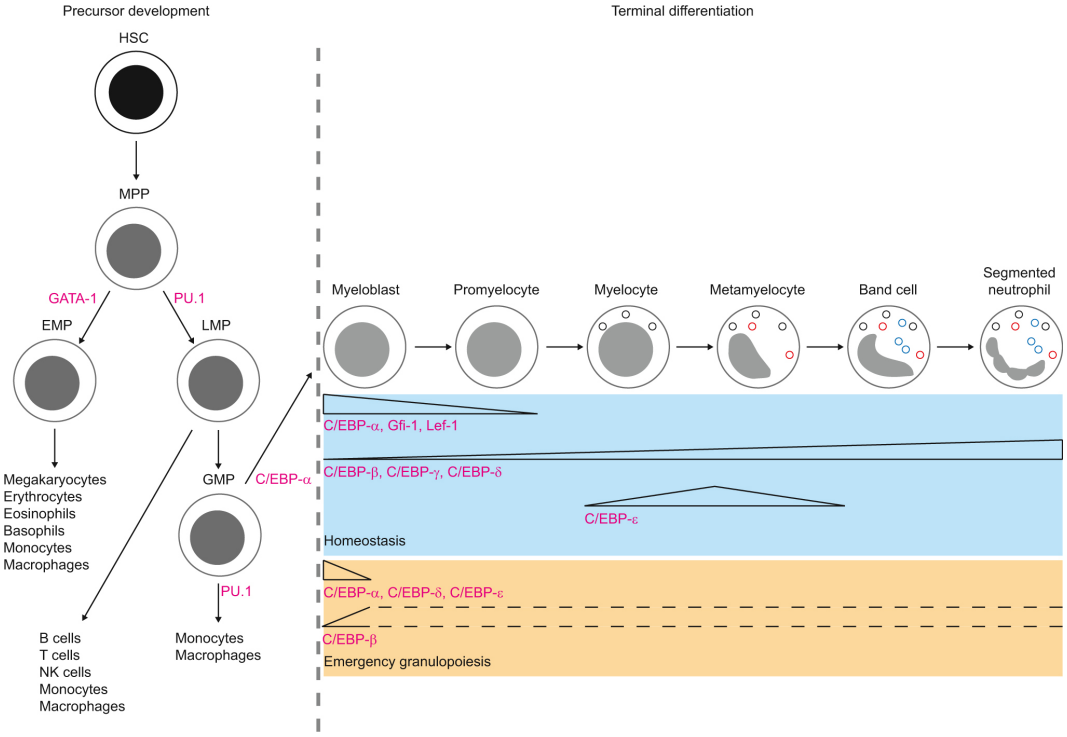


**Figure I-1 | Neutrophil development.** During precursor development hematopoietic stem cells (HSC) give rise to multipotent precursors (MPP) and lymphomyeloid precursors (LMP). Terminal differentiation progresses from myeloblasts via promyelocytes, myelocytes through metamyelocytes and band cells to segmented neutrophils. Granules are sequentially formed: azurophilic granules (black), specific granules (red), gelatinase granules (blue) and secretory granules. Granulopoiesis during homeostasis is dependent on various transcription factors of the C/EBP family, whereas emergency granulopoiesis is mainly driven by C/EBPβ. | Reprinted from Cell Host & Microbe, (Bardoel, 2014), Copyright 2014, with permission from Elsevier.

and decreases during neutrophil development (Bjerregaard, 2003). Next, myeloblasts differentiate into promyelocytes. From this stage onward granules are produced gradually and promyelocytes contain primary (or azurophilic) granules. Secondary (or specific) granule-containing myelocytes develop next. This stage of development is marked by cell cycle exit (Klausen, 2004). From the myelocyte stage to the following metamyelocyte stage, the transcription factor C/EBPε is expressed for a short time. C/EBPε is required for the expression of granule proteins (Bjerregaard, 2003; Morosetti, 1997; Yamanaka, 1997) and C/EBPε mutation leads to the rare condition of specific granule deficiency (Gombart, 2001). Metamyelocytes further differentiate into band cells, containing tertiary (or gelatinase) granules. Finally, segmented neutrophils that contain secretory vesicles leave the BM and enter the blood stream (Bainton, 1971) (**Figure I-1**). They are characterised by a segmented nucleus and contain more than 700 different granule proteins (Rørvig, 2013).

The transcription factor Gfi-1 is required for neutrophil differentiation (Hock, 2003; Karsunky, 2002) and is upregulated during SC commitment to the granulocyte lineage (Velu, 2009). Gfi-1 represses SC genes, which drives differentiation and restricts SC proliferation (Hock, 2004; Horman, 2009). Furthermore, Gfi-1 is an inhibitor of monocytopoiesis. It represses the transcription factor early growth response-2 (Egr2) as well as Csf1, the gene coding for the monocyte-promoting colony stimulating factor 1 (CSF-1) (Zarebski, 2008). Mutations that affect the DNA binding site of Gfi-1 cause severe congenital neutropenia (Person, 2003).

PU.1 activity is important for the initial myelopoiesis commitment and increases during the maturation from the myelocyte stage on. PU.1-deficient mice are impaired in terminal differentiation of granulocytes and have a lack of committed SCs (Bjerregaard, 2003).

C/EBPα expression slowly decreases after the myeloblast stage, whereas C/EBPε is at its highest at the myelocyte-metamyelocyte stage. C/EBPβ,

C/EBPγ, C/EBPδ and C/EBPζ all continuously increase after the metamyelocyte stage up until the end (Bjerregaard, 2003) (**Figure I-1**). C/EBPs form homo- and heterodimers and are additionally regulated by phosphorylation, enabling a highly specific expression of granule proteins during terminal differentiation (Chumakov, 2007).

### *Neutrophil release*

Under normal conditions only mature neutrophils are released from the BM whereas hematopoietic stem cells are retained in BM niches. Therefore, neutrophils express different CXCR motif receptors that regulate retention and release.

BM stromal cells such as osteblasts and vascular endothelial cells produce membrane bound forms of stem cell factor and stromal derived factor 1 (SDF1 or CXCL12), which bind c-kit (CD117) and CXCR4 on neutrophils, respectively. CXCR4 is required for the retention of SCs and neutrophils in the BM and its expression gradually decreases as neutrophils mature (Ding and Morrison, 2013; Ding, 2012; Lapidot and Kollet, 2002). Increased CXCR4 signalling in WHIM syndrome (warts, hypogammaglobulinemia, infections, myelokathexis) causes neutrophil deficiency in the circulation and accumulation of mature neutrophils in the BM (Hernandez, 2003). G-CSF signalling downregulates expression of CXCL12 by BM endothelial cells, allowing for neutrophil egress (Christopher, 2009).

Conversely, during neutrophil maturation expression of CXCR2 increases, allowing neutrophils to respond to the pro-release signals CXCL1 (KC), CXCL2 (MIP-2), CXCL3, CXCL5 and CXCL6 as well as CXCL8 (IL-8) in humans (Hopman and DiPersio, 2014; Martin, 2003). G-CSF upregulates the expression of CXCL1 and CXCL2 on BM endothelial cells, favouring neutrophil release, whereas deletion of CXCR2 leads to retention of mature neutrophils in the BM (Eash, 2009).

*Homeostasis*

Neutrophils are not only important for antimicrobial defence, but are also able to harm the host. Therefore, tight regulation of neutrophil production and release is crucial. At steady state only 1-2% of mature neutrophils are found in circulation in mice. The number of neutrophils in the peripheral tissues influences the production rate of new neutrophils in a negative feedback loop. Low levels of peripheral neutrophils stimulate resident macrophages and DCs to produce IL-23, which in turn activates production of IL-17A by γδ T cells and NK-like T cells (Ley, 2006). IL-17A stimulates production of G-CSF, which enhances neutrophil differentiation (Schwarzenberger, 2000; Stark, 2005). In contrast, abundance of neutrophils in the peripheral tissues leads to neutrophil apoptosis and clearance of the apoptotic cells by macrophages and DCs. This process termed efferocytosis leads to a reduction of the IL-23 production (Ley, 2006; Stark, 2005; von Vietinghoff and Ley, 2009) followed by low levels of IL-17A and G-CSF, causing neutrophil retention in the BM. The importance of IL-17A in this process is still debated since mice that are deficient in IL-17-producing cells can still increase granulopoiesis independent of IL-17 (Bugl, 2012; Bugl, 2013).

Ageing ($CD62L^{lo}$) neutrophils upregulate CXCR4 and home to the BM, where they are ingested by macrophages. The macrophages in turn activate the transcription factor LXR that mediates downregulation of CXCL12 on stromal cells, leading to release of CD62Lhi neutrophils from the BM (Casanova-Acebes, 2013). The renewal of the neutrophil pool peaks every 24 hours and is dependent on the circadian rhythm mediated by the sympathetic nervous system (Scheiermann, 2012).

*Emergency granulopoiesis*

Neutrophils are one of the key cell types in antimicrobial defence and are readily recruited to the site of infection. Since the majority of neutrophils

undergo cell death in the tissue, antimicrobial activity leads to neutrophil consumption. If local infections are not cleared and spread systemically, neutrophil depletion increases. To replenish the neutrophil pool and provide continuously for the high demand of neutrophils during infection, steady state granulopoiesis switches to emergency granulopoiesis (Manz and Boettcher, 2014). This involves the *de novo* generation of neutrophils by increased proliferation of myeloid progenitors as well as the release of mature and immature neutrophils from the BM. Neutrophil depletion upon chemotherapy or irradiation also triggers a compensatory granulopoiesis that is similar to emergency granulopoiesis upon systemic infection.

Increased serum concentrations of microbial products and inflammatory mediators such as IL-6, TNFα, G-CSF, GM-CSF and IL-3 during infection are sensed by tissue macrophages or non-immune cells (Kawakami, 1990; Selig and Nothdurft, 1995; Tanaka, 1996). Emergency granulopoiesis and neutrophil release is dependent on C/EBPβ (Hirai, 2013; Hirai, 2006) (**Figure I-1**) and therefore responsive to G-CSF. However, the absolute requirement for G-CSF during emergency granulopoiesis is dependent on the type of infection. Whereas G-CSF is not required during *C. albicans* infection (Basu, 2000), it is crucial in the context of *Listeria monocytogenes* (Panopoulos and Watowich, 2008). The recognition of microbial components in this context involves signalling through TLR4 and TRIF and is dependent on the microbiome. Strikingly, germ-free mice are severely neutropenic (Bugl, 2013).

The differentiation of neutrophils can also occur at the site of infection. Hematopoietic stem and progenitor cells (HSPC) can migrate to tissues such as skin wounds where they differentiate into mature neutrophils. This process is dependent on TLR2 and MyD88 (Granick, 2013).

### *Extravasation*

For neutrophils to reach the sites of infection, they need to leave the blood vessels and transmigrate through the endothelial wall into the tissue. Transmigration is not only a migration process, but also further primes neutrophils for their subsequent antimicrobial activity, leading to an altered, activated phenotype. Neutrophil transmigration is preceded by a tightly regulated adhesion cascade that is mediated by selectins and integrins. The process is initiated by microbial PAMPs, danger associated molecular patterns (DAMPs) from damaged or dead cells or cellular stress. These signals are integrated by tissue resident macrophages, mast cells and DCs. The subsequent release of pro-inflammatory molecules initiates neutrophil recruitment (Ley, 2007).

The vessel endothelium proximal to the site of infection is activated rapidly (within minutes) by inflammatory molecules such as histamine and platelet activating factor (PAF). This type I activation leads to expression of pre-formed adhesion molecules such as P-selectin, independent of protein synthesis. Type II endothelium activation is slow (hours) and requires *de novo* transcription of selectins, integrin ligands and chemoattractants. It is induced by IL-1β and TNFα (Pober and Sessa, 2007).

Neutrophils arriving in the postcapillary venules close to the site of infection engage in weak and transient interactions with the endothelial cells, leading to reversible capture by the endothelium (**Figure I-2**). Neutrophil glycoproteins such as the P-selectin glycoprotein ligand-1 (PSGL-1) bind to P- and E-selectins on the endothelium. Additionally, free neutrophils can interact with already attached neutrophils via binding of a leukocyte L-selectin to PSGL-1 (Walcheck, 1996). These interactions are stabilised by flattening of neutrophil microvilli, elongation of the rear ends and formation of membrane slings (Chen and Springer, 1999; Sundd, 2012). This process slows the neutrophils down, leading to rolling along the endothelium, and increases the interaction surface for integrins and chemokine receptors

(**Figure I-2**). Rolling neutrophils bind to E-selectin via PSGL-1 and initiate further short-lived and weak bonds to the endothelium: Neutrophil lymphocyte function-associated antigen 1 (LFA-1, integrin αLβ2) and very late antigen-4 (VLA-4, integrin α4β1) bind to endothelial intercellular adhesion molecule 1 (ICAM-1, CD54) and vascular cell adhesion protein 1 (VCAM-1, CD106). These interactions further slow down the rolling, until the neutrophils come to arrest (Zarbock, 2011) (**Figure I-2**). Arrest is initiated by inside-out signalling by chemoattractants via G-protein-coupled receptors (GPCR) on neutrophils. GPCR signalling activates high affinity bonds through Macrophage-1 antigen (Mac-1, integrin αMβ2), LFA-1 and VLA-4 and the endothelial ICAM-1 and VCAM-1 (Alon and Feigelson, 2009; Dixit and Simon, 2012). Adherent neutrophils now crawl along chemotactic cues by serially activating integrins via chemokine-GPCR signalling (Shulman, 2009) (**Figure I-2**). The crawling is regulated by the actomyosin machinery and involves cytoskeletal rearrangements and integrin recycling. Crawling neutrophils display a protrusive leading edge as well as a contractile rear uropod (Hyun, 2012). The serial activation of integrin contacts allows neutrophils to move against or perpendicular to the blood flow direction.

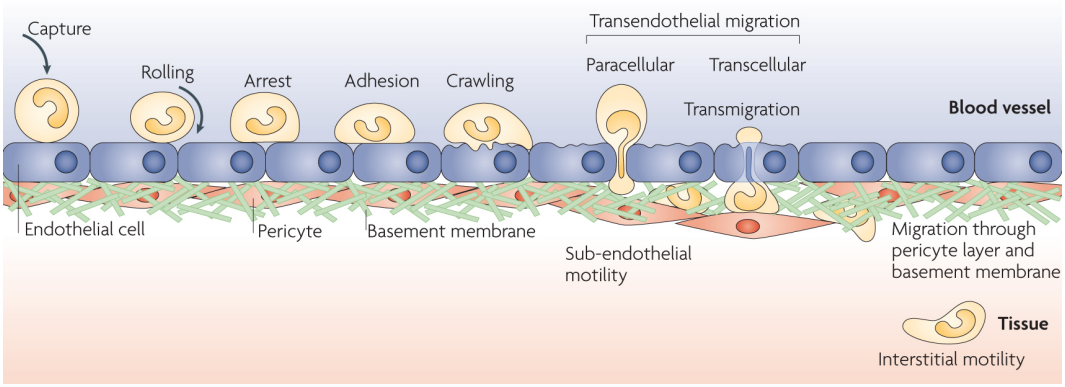


**Figure I-2 | Neutrophil extravasation.** Neutrophil are captured by the endothelial lining, where they roll, arrest, adhere and crawl in an integrin- and selectin- mediated leucocyte adhesion cascade. Finally, neutrophils migrate trough the epithelial layer via a paracellular or a transcellular route. On the abluminal side of the vessel, neutrophils breach through the pericyte sheath and the basement membrane. | Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, (Nourshargh, 2010), copyright 2010.

Finally, neutrophils transmigrate through the endothelium out of the vessel, by extension of protrusions through cell-cell junctions or into endothelial cell bodies (**Figure I-2**). This transendothelial migration (TEM) requires a combination of exit cues, chemotactic guidance, specific endothelial interactions and changes in neutrophil morphology (Nourshargh, 2010). 70-90% of TEM is paracellular and occurs between adjacent cells by breaching cell-cell junctions. Only a small fraction of neutrophils leaves the vessels on the transcellular route, crossing directly through endothelial cell bodies (Ley, 2007; Woodfin, 2011).

Signalling via ICAM-1 and VCAM-1 induces reduction in the endothelial barrier properties, which include increased intracellular $Ca^{2+}$ concentrations, increased ROS production and activation of p38 mitogen-activated protein kinase (MAPK) signalling (Hu, 2000; Huang, 1993; Martinelli, 2009). ICAM-1 activation leads to phosphorylation of cell junction molecules (Allingham, 2007). Junctional structures in the endothelium are mainly adherens junctions that contain VE-cadherin and tight junctions. The expression of adhesion molecules is regulated by inflammatory signals and TEM (Voisin and Nourshargh, 2013). Junctional adhesion molecules can recycle into intracellular compartments (Muller, 2011). Close proximity of adherent leukocytes induces the transient loss of surface VE-cadherin by reversible endocytosis (Wessel, 2014). Accordingly, leukocyte transmigration is inhibited in mice with defects in VE-cadherin endocytosis and turnover (Broermann, 2011; Vestweber, 2012).

Transcellular TEM is induced by ICAM-1 signalling and its translocation to actin- and caveola-rich domains. Formation of vesiculovacuolar organelles (VVOs) and intracellular channels allows neutrophils to breach through the endothelial cells via a transcellular pore (Carman, 2007; Millan, 2006).

To leave the vessel walls neutrophils need to exit on the abluminal side and cross through the pericyte sheath and the venular basement membrane (Nourshargh, 2010). Pericytes are irregularly wrapped around the

endothelial cells and embedded in the basement membrane. They express adhesion molecules, chemokines and receptors for pro-inflammatory cytokines (Pober and Tellides, 2012; Stark, 2013; Voisin and Nourshargh, 2013). Pericytes provide a matrix for neutrophils to crawl out of the venular wall, dependent on interactions between pericyte ICAM-1 and neutrophil Mac-1 and LFA-1 (Proebstl, 2012). During TEM, neutrophils are specifically primed for this interaction with pericytes (Ayres-Sander, 2013). How neutrophils breach the basement membrane is not completely understood, but leukocyte-permissive regions may exist that have low abundance of matrix proteins. These permissive regions are termed low expression regions (LERs) and are located in the proximity of paracellular exit sites (Voisin and Nourshargh, 2013; Voisin, 2010).

### *Neutrophils in tissues*

Neutrophils follow a chemokine gradient to extravasate from the blood vessels. However, in order move away and to migrate further into the tissues they need to overcome this gradient. Interestingly, a hierarchy of chemotactic gradients exists. Intermediate chemoattractants such as CXCL8 are released by the endothelium. End-target chemoattractants such as fMLP and complement C5a in the immediate proximity of bacteria override intermediate chemoattractants. Neutrophils preferential respond to end-target chemoattractants (Foxman, 1997). End-target chemoattractants signal via different pathways for activation and inhibition of neutrophil recruitment. Low concentrations of fMLP favour neutrophil migration via the p38 MAPK-pathway. In contrast, high fMLP concentrations inhibit neutrophil migration in an ERK-dependent manner (Liu, 2012).

In the tissues, neutrophils induce the generation of chemokines and pro-inflammatory cytokines in mesenchymal and myeloid cells, leading to recruitment of other inflammatory cells and more neutrophils. Neutrophils can also directly attract more neutrophils to the tissues via release of leukotriene B4 (LTB4). The first wave of neutrophils migrate towards the

centre of the inflammation and high concentrations of LTB4. This attracts a second "swarm" of neutrophils expressing the leukotriene receptor (Lammermann, 2015; Lammermann, 2013). Such chemokine signalling makes migration over long distances possible.

Additionally, neutrophils can migrate out of the vasculature and persist as tissue resident cells. Uninfected lungs have a marginated neutrophil pool, which consists of neutrophils that reside adjacent to the luminal endothelial surface. During infection monocytes induce extravasation of these neutrophils into the tissue (Kreisel, 2010).

Inflammation is triggered both in response to infection and to sterile injury caused by for example burns, hypoxia, or chemicals. Different PAMPs and DAMPs can activate the same PRRs and lead to similar pro-inflammatory responses. The formyl peptide receptor (FPR) responds to formylated peptides released during thermal injury from mitochondria of damaged cells or from bacteria during infection (McDonald, 2010). During infection, neutrophils are required for resolution and protective immunity. However, during sterile inflammation neutrophils aggravate inflammation by releasing oxidants, proteases and antimicrobial proteins, causing bystander injury. It is not clear whether the neutrophils in both scenarios differ and whether neutrophils can distinguish between infection and sterile inflammation. Possibly pro-inflammatory and pro-resolving neutrophils are recruited.

### 1.1.2 Neutrophil immune responses

In the tissues neutrophils encounter a large variety of different microbes. Neutrophils posses a range of antimicrobial strategies such as degranulation, phagocytosis and NET release, but it is unknown whether neutrophils tailor their antimicrobial strategies to fight fungi selectively. Neutropenia and deficiencies in neutrophil antimicrobial strategies, lead to an increased incidence of invasive fungal infections, indicating the importance of neutrophils in antifungal immunity (Brown, 2011).

## *Microbial sensing*

Neutrophils are equipped with an array of pattern recognition receptors (PPRs) that allow them to sense microbes directly. They recognise bacterial, viral and fungal PAMPs. The activation state and life span of neutrophils depends largely on the signals received from cytokines and PAMPs. The PRRs expressed on neutrophils include membrane bound receptors such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic receptors such as absent in melanoma 2 (AIM2), retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation antigen 5 (MDA5) and intracellular nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) (**Figure I-3**). Polymorphisms in PRRs or adaptor molecules leading to abrogated signalling pathways in neutrophils and other innate immune cells often cause increased susceptibility to fungal infections in the affected tissues.

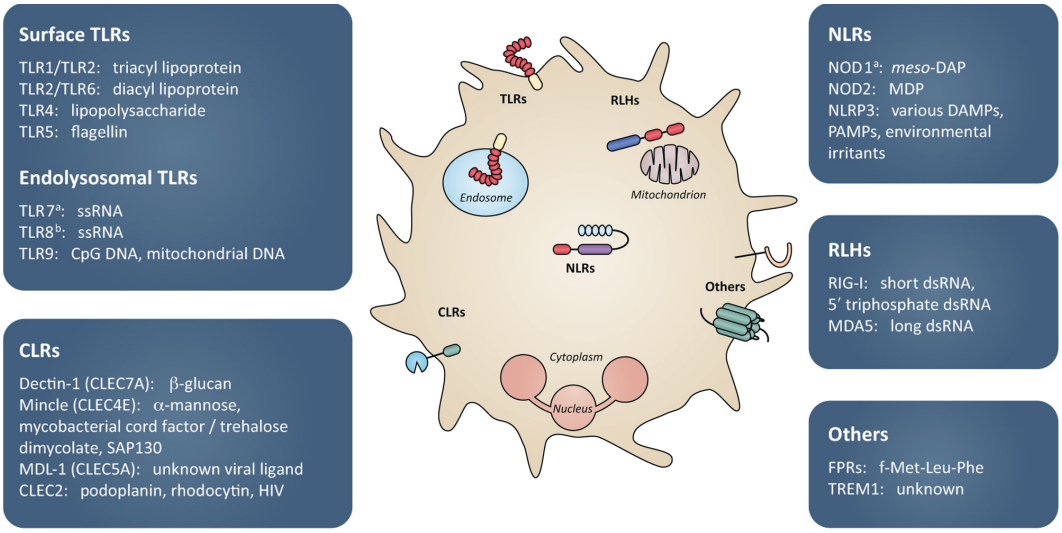


**Figure I-3 | Neutrophil pattern recognition receptors (PRRs) and ligands.** The main neutrophil PRR families are TLRs, CLRs and NLRs. While TLRs and CLRs recognise microbial pathogen-associated molecular patterns (PAMPs), NLRs signal upon ligation by danger-associated molecular patterns (PAMPs). For details see the main text. | Abbreviations: CLEC, C-type lectin domain family; CLR, C-type lectin receptor; meso-DAP, meso-diaminopimelic acid; FPR, formyl peptide receptor; MDL, myeloid DAP12-associating lectin; NLR, nucleotide-binding oligomerisation domain (NOD)-like receptor; NLRC, NLR with a CARD domain; NLRP, NLR with a pyrin domain; RLH, retinoic acid-inducible gene (RIG)-like helicase; SAP, spliceosome-associated protein; TLR, Toll-like receptor; TREM, triggering receptor expressed on myeloid cells. $_{a}$Mouse but not human neutrophils. $_{b}$Human but not mouse neutrophils. | Reprinted from Trends in Immunology, (Thomas and Schroder, 2013), Copyright 2013, with permission from Elsevier.

*Toll-like receptors (TLRs)*

TLRs are expressed on the cell surface and in the phagosomal membrane of innate immune cells. TLR signalling leads to activation of the transcription factors NK-κB, activator protein 1 (AP-1) and the interferon regulatory factor (IRF) family and triggers the release of pro-inflammatory cytokines and type I interferons (IFN). Neutrophils express nearly all TLRs except for TLR3 and TLR7 in humans (Hayashi, 2003; Tamassia, 2008). TLR7 mRNA is expressed in mouse neutrophils (Charmoy, 2007) that respond to the TLR7 ligand R848 after priming with GM-CSF (Wang, 2008). TLRs are involved in the regulation of nearly all neutrophil responses, including migration, phagocytosis, degranulation, ROS production, apoptosis, NETosis and cytokine release. TLR signalling also strongly synergises with other stimuli. Activation of TLR4 by bacterial lipopolysaccharide (LPS) enhances ROS production upon stimulation with fMLP (Parker, 2005; Prince, 2011). As opposed to macrophages, neutrophil TLRs signal solely via the adapter protein myeloid differentiation primary response gene 88 (MyD88) and not via TIR-domain-containing adapter-inducing interferon-β (TRIF). Consequently, neutrophil TLR signalling does not induce the production of type I IFN. TRIF is poorly expressed in human neutrophils and it is unclear whether it is expressed in mouse neutrophils (Tamassia, 2007; van Bruggen, 2010). Interestingly, MyD88-dependent TLR signalling seems to be dispensable in some cases. Mice deficient in the MyD88-dependent interleukin-1 receptor-associated kinase 4 (IRAK4) are unaffected in their ability to kill *C. albicans, E. coli* and *S. aureus*, indicating redundant PRR signalling independent of TLR (van Bruggen, 2010).

The contribution of TLR signalling to antifungal immunity in neutrophils and other innate immune cells varies depending on the fungal species, morphotypes, route of infection and receptor cooperativity. TLR2, TLR4 and TLR6 are the main TLRs involved in fungal recognition and bind zymosan, phospholipomannan and O-linked mannans (Netea, 2008; van de Veerdonk, 2008). Polymorphisms in TLR4 are associated with increased susceptibility

to pulmonary aspergillosis and bloodstream candidiasis in humans (Bochud, 2008; Carvalho, 2008; Van der Graaf, 2006). Furthermore, intracellular TLRs are involved in antifungal immunity. TLR9 recognises fungal DNA and chitin followed by the production of anti-inflammatory cytokines (Wagener, 2014). Polymorphisms in TLR9 are associated with allergic bronchopulmonary aspergillosis in humans (Carvalho, 2008). TLR signalling also induces cellular antifungal immunity via the production of type I interferons, pro-inflammatory TNF-α and IL-12, promoting adaptive immunity (Bourgeois, 2011; Carvalho, 2012; Ramirez-Ortiz, 2008). Furthermore, TLRs mediate the presentation of fungal antigens by DCs to T cells and control antigen processing and presentation (Blander and Medzhitov, 2006). TLRs are found to cooperate with CLRs and modulate CLR signalling (Netea, 2006). TLR2 and dectin-1 trigger phagocytosis and cytokine production in mice and deficiency of the CLR adaptor CARD9 leads to increased fungal infections in humans (Drummond and Brown, 2011; Glocker, 2009). Interestingly, the role for TLRs differs in humans and mice. In mice deficiency of the TLR adaptor MyD88 leads to increased susceptibility towards *C. albicans*, *A. fumigatus* and *C. neoformans,* whereas in humans MyD88-deficiency causes susceptibility to bacterial but not fungal infections (Biondo, 2005; Bretz, 2008; Marr, 2003; von Bernuth, 2008).

*C-type lectin receptors (CLRs)*

CLRs are $Ca^{2+}$-dependent carbohydrate-binding receptors with one or more structural C-type lectin-like domain (CTLD) (Osorio and Reis e Sousa, 2011). They signal through intracellular immunoreceptor tyrosine-based activation motifs (ITAM), hemi-ITAMs or associate with ITAM-containing adaptors. Some CLRs posses immunoreceptor tyrosine-based inhibitory motifs (ITIM). CLR activation triggers phosphorylation of ITAMs and activation of the spleen tyrosine kinase (Syk). Syk signalling regulates neutrophil phagocytosis and ROS through the activation of NF-κB, MAPK and nuclear factor of activated T cells (NFAT) (Mócsai, 2010). The canonical NF-κB-

caspase recruitment domain-containing protein 9 (CARD9) pathway is important for antifungal responses (Gross, 2006) and deficiencies in CLRs cause increase susceptibility to fungal infections.

Dectin-1

Dectin-1 (CLEC7A) is primarily expressed by myeloid cells. It recognises β-1,3-linked and β-1,6-linked glucans in a variety of fungal species including *C. albicans*, *Aspergillus fumigatus* and *Pneumocystis carinii.* Dectin-1 is both a phagocytic and a signalling receptor.

Activation of dectin-1 induces production of pro- and anti-inflammatory cytokines and chemokines. Receptor ligation leads mainly to activation of the Syk-CARD9 signalling pathway through protein kinase C (PKC) δ and RAF1 kinase (Drummond and Brown, 2011; Drummond, 2011; Glocker, 2009; Gross, 2006; Strasser, 2012). Dectin-1-induced signalling pathways act synergistically and cross regulatory and induce canonical and non-canonical NF-κB activation. Syk-CARD9 pathway activation induces cytokine production, ROS burst and inflammasome activation (Hardison and Brown, 2012). Dectin-1 activates IL-1β production via the NOD-, LRR- and pyrin domain-containing 3 (NLRP3) inflammasome and caspase 1 (Cheng, 2011) or via a non-canonical caspase 8 inflammasome (Gringhuis, 2012). On neutrophils, dectin-1 signalling is CD11b-dependent. CD11b binds β-glucan and signals via Syk through the ITAM-containing adaptors FcRγ and DNAX activation protein of 12kDa (DAP12) (Mócsai, 2006; van Bruggen, 2009).

Deficiencies in dectin-1 and CARD9 are associated with increased fungal susceptibility. Dectin-1-deficient mice show increased mortality upon infection with *C. albicans* (Taylor, 2007) and *A. fumigatus* (Werner, 2009) and have increased fungal burdens upon infection with *P. carinii* (Saijo, 2007) and *Coccidioides* (Viriyakosol, 2013) species. Polymorphisms in human dectin-1 trigger recurrent vulvovaginal candidiasis including colonisation of the urinary tract (Ferwerda, 2009) and increased invasive aspergillosis (Cunha,

2010), but show no effect on systemic candidiasis (Smeekens, 2013). However, CARD9 deficiency in mice and mutations in humans lead to increased susceptibility for invasive candidiasis (Drewniak, 2013; Glocker, 2009; Gross, 2006). Differences in β-glucan expression and structure between different fungal strains may account for strain-dependent susceptibility to infection.

Dectin-1 mediated activation of phagocytosis by particulate β-glucan occurs via the formation of a phagocytic synapse in macrophages. Since neutrophils do not respond to soluble β-glucan, the existence of a similar signalling synapse in neutrophils is likely (Goodridge, 2011).

### Mannose receptor

The mannose receptor (MR) recognises a broad range of microbes of all classes, including fungi mainly via N-linked mannans (Lam, 2007). MR also has affinities for fungal α-glucans and chitin. Due to the lack of a signalling motif in the cytoplasmic tail, the MR forms heterodimers with TLRs, dectin-1 and the peroxisome proliferator activated receptor-γ (PPARγ) (Gales, 2010). MR activation leads to production of IL-17 and phagocytosis of unopsonised yeast. However the role of MR in antifungal immunity is mainly redundant with other receptors (Hardison and Brown, 2012).

### Dectin-2

CLEC6A is the most important member of the dectin-2 family. It is expressed on DCs, macrophages and neutrophils (Saijo, 2010). The receptor recognises high mannose-containing structures like α-mannose and mannose-rich glycoproteins on fungi such as *C. albicans*, *P. brasiliensis*, *A. fumigatus* and *Malassezi* (Ishikawa, 2013; Kerscher, 2013) and binds *C. albicans* hyphae with higher affinity than yeast particles. Dectin-2 has an extracellular carbohydrate recognition domain but lacks an intracellular signalling domain. It pairs with other signalling receptors such as the FcRγ or dectin-3.

The activation of the Syk-CARD9 pathway leads to induction of pro-inflammatory cytokines, ROS production, leukotriene release and $CD4^+$ T cell Th17 polarisation (Drummond, 2011; Hardison and Brown, 2012; Robinson, 2009). Dectin-2-deficient mice are more susceptible to *C. albicans* infection with reduced survival and increased fungal burden but have no phenotype upon infection with *C. neoformans* (Saijo, 2010).

### Mincle

Mincle is another member of the dectin-2 family lacking an intracellular signalling domain. Like dectin-2 it associates with FcRγ and induces the Syk-CARD9 signalling pathway leading to NF-κB-mediated cytokine production and modulation of the adaptive immunity. Mincle recognises damaged cells as well as fungi such as *C. albicans* and *Malassezia* species (Wells, 2008; Yamasaki, 2009). The *C. albicans* ligand is unknown whereas recognition of *Malassezia* happens via glycero-glycolipid mannitol-linked fatty acids (Ishikawa, 2013). Even though mincle is expressed mainly on macrophages it also plays a role in neutrophil signalling (Vijayan, 2012).

#### *Complement receptor 3*

The complement receptor 3 (CR3, also Mac-1 or $\alpha_M\beta_2$) is an integrin, built from a CD11b ($\alpha_M$) chain and a CD18 ($\beta_2$) chain. CR3 is part of the complement system and can act in combination or without other complement factors. CR3 engagement induces phagocytosis, leukocyte adhesion and chemotaxis (Sandor, 2013) as well as complement-independent antibody-dependent phagocytosis and killing of unopsonised *C. albicans* (Gazendam, 2014; Taborda and Casadevall, 2002). CR3 may act as a direct phagocytic receptor for β-glucan, and might account for dectin-1-independent β-glucan responses in humans, which are not observed in mice (van Bruggen, 2009).

*NOD-like receptors (NLRs) and inflammasomes*

NLRs are cytoplasmic receptors that recognise mainly bacterial peptidoglycan. NOD1 and NOD2 activate MAPK and NF-κB signalling. NLRP3, NLRP1 and NLRC4 are part of inflammasome complexes and induce inflammation.

NOD1 and NOD2 recognise different substructures of bacterial peptidoglycan. They activate NF-κB and MAPK signalling via interaction of the CARD domain with the kinase receptor-interacting serine/threonine protein kinase 2 (RIP2) (Fritz, 2006). NOD1 is not found in human peripheral neutrophils (Ekman and Cardell, 2010), but induces signalling in murine BM neutrophils (Clarke, 2010). Gut microbiome-derived peptidoglycan signals constitutively through NOD1 and primes BM neutrophils for phagocytosis and ROS production (Clarke, 2010). NOD2 is expressed in mouse and humans (Ekman and Cardell, 2010). Its activation leads to loss of CD62L and CD11b upregulation, chemokine production and neutrophil migration (Ekman and Cardell, 2010). NLRs are involved in fungal sensing and NOD2 in combination with MR and TLR9 recognise chitin and stimulate the production of IL-10 (Wagener, 2014).

Pyrin-containing NLR (NLRP) are one of several inflammasome classes (Latz, 2013). NLRP inflammasome sensors include NLRP3, NLRP1, NLRC4, NLRP12 and AIM2. They consist of a C-terminal leucin-rich repeat (LRR), a central oligomerisation domain and a N-terminal effector domain. Inflammasomes are cytosolic sensors of DAMPs and control the maturation of IL-1β and IL-18 through caspase 1 (Latz, 2013). IL-1β is an inflammatory cytokine that acts locally and systemically and requires a two-step activation. Signalling via dectin-1 or TLR2/TLR4 upregulates transcripts of the pro-form of IL-1β (Bellocchio, 2004; Hise, 2009; Netea, 2002). A second inflammasome-dependent proteolytic step by caspase 1 is required to cleave IL-1β into its active form (Wilson, 1994). The NLRP3 inflammasome is the best-studied inflammasome and is expressed in neutrophils, macrophages, T cells, B cells,

DCs and mucosal epithelium. It responds to a variety of stimuli that include microbes (Allen, 2009; Craven, 2009; Dostert, 2009), host molecules such as ATP (Mariathasan, 2006), uric acid crystals (Martinon, 2006), amyloid-β fibrils (Halle, 2008) and crystalline compounds such as silica crystals, asbestos and aluminium salts (Dostert, 2008; Hornung, 2008). Purified mouse neutrophils activate Il-1β production upon stimulation with the NLRP3 agonist nigericin or upon *S. aureus* infection (Mankan, 2012). Mutations in NLRP3 and NLRP12 are associated with IL-1β-dependent hereditary periodic fever syndrome (Jéru, 2008). Defective NLRP3 inflammasome activation leads to increased colonisation with *C. albicans* (Gross, 2009) and increased susceptibility to mucosal candidiasis and disseminated disease (Hise, 2009). The inflammasome activating *C. albicans* ligands are not entirely defined, but the cell wall preparations such as zymosan, curdlan or the cell wall component mannan and the secreted aspartic proteases Sap2 and Sap6 were all shown to activate NLRP3 (Kumar, 2009; Lamkanfi, 2009; Pietrella, 2013). Interestingly, *A. fumigatus* and *C. albicans* hyphae but not yeast activate NLRP3, possibly providing a means of discrimination between colonisation and invasive state of the fungus (Gow, 2012; Joly, 2009).

Even though inflammasomes are the main route of IL-1β activation, the cytokine can also be processed by monocytes that express constitutively active caspase 1 (Netea, 2009) or by neutrophil PR3 (Netea, 2015b). Since neutrophils are the main effector cells in the early response, IL-1β processing by PR3 plays an important role in the early stages of antifungal immunity. Interestingly, PR3 and not caspase 1 is crucial for protection against disseminated candidiasis in mice (Mencacci, 2000). Additionally, *C. albicans*-derived proteases can process pro-IL-1β (Beausejour, 1998).

## *Anti-microbial strategies*

Neutrophils posses a range of antimicrobial strategies including release of antimicrobials from their granules, phagocytosis, production of reactive oxygen and NETosis.

### *Degranulation*

Neutrophils contain four classes of granules comprised of azurophilic granules, specific, gelatinase and secretory granules (Borregaard and Cowland, 1997). Neutrophils granules are heterogeneous in protein content and exocytosis capacity. The expression profile of the granule proteins changes during neutrophil precursor maturation. Since granule proteins do not contain sorting signals for individual granule compartments, their distribution is solely dependent on timing (Le Cabec, 1996). Azurophilic granules are formed first during the myeloblast and promyelocyte stages and contain a range of proteases such as NE, CG and PR3 as well as antimicrobial proteins such as defensins, lysozyme and MPO. Next, during the myelocyte/metamyelocyte stage specific granules are formed. They contain proteins such as collagenase, gelatinase, lactoferrin and increasing amounts of lysozyme. Gelatinase granules are formed during the band cell stage and contain gelatinase, arginase and increasing amounts of integrins including CD11b and CD18 as well as NADPH oxidase components such as $gp91^{phox}$ and $p22^{phox}$. Finally, secretory vesicles are formed last and contain a large variety of receptors including cytokine- (IFN$\alpha$R, IFN$\gamma$R, TNFR) and chemokine receptors (CXCR1, CXCR2, CXCR4) and TLRs, CD14 and MyD88.

Furthermore, the propensity of granules for exocytosis varies. Granules that are formed at late stages during granulopoiesis, such as gelatinase granules, are mobilised first, whereas azurophilic granules are released last (Kjeldsen, 1992; Sengelov, 1995; Sengelov, 1993). The secretion propensity is defined by the density of vesicle-associated membrane protein 2 (VAMP-2) proteins in the granule membrane. VAMP-2 is a fusogenic protein of the v-SNARE (N-

methylmaleimide-sensitive factor attachment protein (SNAP)–SNAP receptor) family. The concentration of v-SNAREs is highest in gelatinase and secretory vesicles (Brumell, 1995) the mRNA profile of VAMP-2 increases during myelopoiesis when neutrophils mature to band cells (Theilgaard-Monch, 2005). Triggers such as the increase of the intracellular $Ca^{2+}$ concentration activate granule exocytosis. Granule membranes are reservoirs of pre-formed proteins. Fusion of the membranes recruits proteins into the plasma membrane, including β2 integrins, NADPH oxidase components, fusogenic proteins such as SCAMP (secretory carrier membrane protein) and receptors such urokinase-type plasminogen-activating receptor and the formyl peptide receptor (Borregaard, 2007; Brumell, 1995; Nauseef, 2007; Nordenfelt and Tapper, 2011). Specific granules fuse with the plasma membrane, whereas azurophilic granules fuse preferentially with the phagosome.

The release of granule contents such as defensins, bactericidal-permeability-increasing protein, azurocidin, MPO, as well as proteases such as NE and cathepsin G creates an antimicrobial environment (Nauseef, 2007). Furthermore, neutrophil granules contain membrane disrupting antimicrobials such as α-defensin or cathelicidin (Kruger, 2015). Lactoferrin and lipocalin inhibit the microbial iron uptake. Lysozyme cleaves cell wall peptidoglycans, whereas proteases such as NE degrade virulence factors and toxins (Weinrauch, 2002). Proteinase-3 (PR3) cleaves the antimicrobial peptide LL-37 into its active form (de Haar, 2006). NE and cathepsin G are activated through N-terminal cleavage by dipeptidyl peptidase 1 (DPPI) and mutations of DPPI are associated with severe neutrophil deficiencies (Korkmaz, 2010). Antimicrobial proteins are also associated to NETs, facilitating the direct interaction with the microbes (Bardoel, 2014).

Degranulation is an important part of neutrophil antimicrobial immunity, as illustrated by deficiencies in specific granules, which causes increased susceptibility to infection (Gombart, 2001). However, the release of

antimicrobials into the environment also has the potential to harm host tissues. Therefore, the process is tightly regulated.

*Phagocytosis*

Phagocytosis is one of the main neutrophil antimicrobial strategies. It is an active process that leads to the uptake of small particles and microbes into an intracellular, membrane-enclosed phagosome. It is a three-step process that is initiated by binding of a ligand-bearing particle, followed by clustering of the phagocytic receptors. Subsequently, receptor signalling, triggers actin-dependent rearrangement of the cytoskeleton, leading to uptake of the particle.

Phagocytosis is initiated through FcR-engagement by opsonised microbes, complement activation or through a large array of PRR that recognise microbes directly, including TLRs, NLRs and CLRs such as dectin-1 (Bardoel, 2014; Kruger, 2015; Nauseef, 2007). In a "zippering" mechanism, receptor interaction with the ligands repeats many times, wrapping the membrane around the particle (Griffin, 1975). The FcγR is the best-studied phagocytic receptor. FcγRIIa (CD32), FcγRIIIb (CD16) and FcγRIV are constitutively expressed in neutrophils, whereas FcγRI (CD64) expression requires G-CSF priming (Nimmerjahn, 2008). Clustering of the FcγR is required for signalling, bringing the cytosolic ITAM motifs of the receptor in close proximity (Holowka, 2007). Upon receptor engagement both tyrosines of the ITAM motifs are phosphorylated by tyrosine kinases of the Src family: Hck, Lyn and Fgr (Hamada, 1993; Wang, 1994). Syk kinase binds to the double phosphorylated ITAMs via its two Src homology 2 (SH2) domains, leading to phosphorylation and activation of Syk (Johnson, 1995). Subsequently, adapter proteins that bind to the receptor complex function as platform for recruitment of downstream signalling components. FcγR-dependent phosphorylation of ITAM motifs leads to elevation of intracellular calcium concentrations, the activation of the NADPH oxidase, actin-polymerisation and granule secretion (Nimmerjahn, 2008). During the phagocytic process a

PI3K-dependent change of the lipid composition in the plasma membrane is required for invagination and phagosome maturation. Furthermore, GTPases of the Rho family are crucial for phagocytosis. In their active, guanosine triphosphate (GTP)-bound form they are anchored to the membrane bilayer of the plasma membrane, whereas in their inactive, guanosine diphosphate (GDP)-bound form are targeted to the cytoplasm. Guanine nucleotide exchange factors (GEF) catalyse the GDP release and GTP replacement. The GTPase Cdc42 is responsible for the formation of filopodia, whereas Rac1 and Rac2 mediate the formation of lamellipodia (Hoppe and Swanson, 2004). The rearrangement of the cytoskeleton is dependent on actin polymerisation. In a process called treadmilling actin monomers are associated at one end of an actin filament and dissociated from the rear end. The nucleator complex Arp2/3 triggers branching of new strands from existing actin filaments. Arp2/3 is activated by nucleation-promoting factors such as the Wiskott-Aldrich syndrome protein (WASP), which is a downstream effector of Cdc42 or the Scar/WAVE proteins, which are effectors of Rac (May, 2000). Actin is cleared from the base of the phagocytic cup, allowing the sinking in of the phagocytosed particle (Larsen, 2002). The needed membrane material is mostly derived from the plasma membrane (Touret, 2005) or delivered from intracellular compartments.

Dectin-1-mediated phagocytosis is similar to FcγR-dependent phagocytosis even though as opposed to the FcγR, dectin-1 possesses only a hemi-ITAM. Dectin-1 also signals via Syk and two dectin-1 receptors are bridged by one Syk molecule (Fuller, 2007). Like in FcγR-mediated phagocytosis PI3K, Cdc42 and Rac1 activation as well as actin remodelling are required (Herre, 2004).

Neutrophil phagocytosis differs in many aspects from that in macrophages. Phagocytosis in neutrophils is extremely fast and internalisation of opsonised targets occurs in less than 30 seconds (Segal, 1980), whereas this process takes several minutes in macrophages. Phagosomes in macrophages mature gradually through the endosomal pathway. In contrast, in neutrophils pre-formed granules fuse with the phagocytic vacuole to form a phagolysosome

(Lee, 2003; Segal, 1980). Azurophilic and specific granules fuse with the plasma membrane, just before the phagosome seals completely. Additionally, azurophilic granules also fuse with the formed phagosome later during the maturation process and are targeted by the microtubule network to the phagosome membrane (Tapper, 2002). The targeting and fusion of neutrophil granules to phagosome is calcium-dependent, but differs between the granule types. Early secretion of azurophilic granules is calcium-dependent, whereas fusion of azurophilic granules with the fully formed phagosome does not require calcium (Nordenfelt, 2009). Macrophage phagosome maturation is calcium-independent.

*ROS production*

Phagocytosis or the activation with soluble agents like PMA or fMLF triggers the production of ROS. The NADPH oxidase assembles first at the plasma membrane and later at the phagosome (Babior, 2004; Babior, 2002; Decoursey and Ligeti, 2005). ROS production in neutrophils exceeds that in macrophages by far, through the recruitment and assembly of additional NADPH oxidase in the phagosomal membrane (VanderVen, 2009). The multicomponent complex is separated in two compartments in resting neutrophils. The flavocytochrome $b_{558}$ is an integral membrane protein that is mainly present in specific granules, but also in the plasma membrane and secretory vesicles. It is a heterodimer formed of the subunits $gp91^{phox}$ (NOX2) and $p22^{phox}$ (Borregaard and Tauber, 1984). Upon activation, the cytosolic components $p47^{phox}$, $ph67^{phox}$, $p40^{phox}$ associate with the complex bound to the membrane (Babior, 2004; Nauseef, 1991; Wientjes, 1993). The GTPases Rac-1 and Rac-2 are also required for complete activation (Vignais, 2002). Neutrophil activation phosphorylates $p47^{phox}$, leading to a conformational change, translocation to the membrane and association with flavocytochrome $b_{558}$ (Berthier, 2003; Heyworth, 1991). During phagocytosis $p47^{phox}$ and $p67^{phox}$ are recruited to the phagosome where they remain transiently. $p40^{phox}$ associates with $p67^{phox}$, a requirement for its

translocation to the phagosome (Allen, 1999; Faure, 2013; van Bruggen, 2004).

The activated NADPH oxidase complex transfers electrons from NADPH in the cytosol through membrane-bound flavocytochrome $b_{558}$ onto molecular oxygen in the phagosome, reducing it to superoxide anion. The ROS burst consumes protons, which counteracts the acidification of the neutrophil phagosome, maintaining a neutral pH. (Jankowski, 2002). A range of different ROS is formed through spontaneous dismutation of the superoxide and action of MPO. Furthermore, oxidants diffuse from the phagosome and fuse with other granule molecules. ROS derivatives such as hydrogen peroxide, hydroxyl radicals, hypochlorous acid, singlet oxygen or ozone are formed during this process (Klebanoff, 2005). Different ROS have specific activities and through the collaboration of oxidants and granule proteins the phagocytosed microbes are killed.

ROS production is important for microbe killing and mutation in any of the NADPH oxidase subunits causes chronic granulomatous disease (CGD). Oxidase-defective CGD neutrophils have greatly impaired antimicrobial function. Killing of *S. aureus* can be restored in CGD neutrophils by glucose oxidase (GO)-loaded liposomes or PEG-D-amino acid oxidase, which produces $H_2O_2$ (Gerber, 2001; Nakamura, 2012). Interestingly, NADPH oxidase-inhibition does not impair killing of *S. pneumoniae, E. coli* or *P. aeruginosa* (Standish and Weiser, 2009), indicating that other antimicrobial strategies are in place. However, although some microbes are still killed, NADPH oxidase deficiency greatly impairs neutrophil function.

*Neutrophil extracellular traps (NETs)*

NETs are web-like structures formed of extracellular chromatin and neutrophil antimicrobial molecules. NETs are released in a form or programmed cell death termed NETosis. NETs trap and kill a variety of

microbes, complementing the neutrophil antimicrobial defence by an extracellular strategy.

The formation of NETs and the impact on neutrophil immunity is discussed in detail in the following chapter. See **NETosis** on page 51.

### *Immune cell interaction*

Neutrophils interact with a number of innate and adaptive immune cells including DCs, macrophages, NK cells, T cells (Scapini and Cassatella, 2014).

During infection with *Leishmania*, DCs capture phagocytosing and apoptotic neutrophils. This *Leishmania*-driven mechanism undermines the efficient immune clearance of the parasite (Ribeiro-Gomes, 2012). Neutrophils themselves can acquire DC characteristics and express CD11c, MHCII and the T cell co-stimulatory molecules CD80 and CD86. This plasticity is driven by GM-CSF that is also responsible for prompting macrophages to acquire a DC phenotype (Geng, 2013; Matsushima, 2013). Neutrophils can migrate to the lymph nodes upon activation of their CCR7 receptor by lymph node-secreted CCL19 and CCL21 (Beauvillain, 2011). In the lymph nodes neutrophils produce thromboxane $A_2$, which reduces the contact between DCs and T cells. Thromboxane $A_2$-mediated vessel constriction inhibits the migration of T cells to more central lymph nodes (Yang and Unanue, 2013). Neutrophils are the main producers of MPO. MPO can inhibit the proliferation and activation of DCs, restricting $CD4^+$ T cell activity in the lymph nodes (Odobasic, 2013).

Furthermore, neutrophils interact in various ways with the T cell compartment. Th17 cells induce neutrophil recruitment by production of IL-17 and CXCL8. In turn, neutrophils produce CCL20 and CCL2, mediating Th17 recruitment in a positive feedback loop (Pelletier, 2010). During systemic inflammation a subset of activated $CD62L^{dim}$ neutrophils suppresses T cell proliferation in a Mac-1-dependent manner (Pillay, 2012). Neutrophils that come from the periphery can home back into the BM in a CXCR4-dependent

manner. They can bring with them antigen from the dermis to the BM, bypassing the lymph nodes. Resident macrophages in the BM take the neutrophils up and present the antigen to naive $CD8^+$ T cells promoting their differentiation into memory T cells (Duffy, 2012). Finally, neutrophilic myeloid-derived suppressor cells (MDSCs) interact with T cells through ROS, indoleamine-2,3-dioxygenase (IDO), Mac-1, programmed death-ligand 1 (PD-L1) and STAT3 to inhibit T and NK cell proliferation (Nagaraj, 2013; Youn and Gabrilovich, 2010).

Furthermore, neutrophils are involved in NK cell maturation and neutropenia causes diminished NK cell activity (Jaeger, 2012).

In the spleen marginal zone neutrophils secrete the B cell and plasma cell survival factors B cell activating factor (BAFF) and A proliferation-inducing ligand (APRIL) (Huard, 2008; Scapini, 2005). In turn, a subset of splenic B-cells produces GM-CSF, leading to differentiation of splenic neutrophils (Cerutti, 2013). A study described "B-cell helper neutrophils" in the spleen that activate marginal zone B cell class switching and T-independent antibody production (Puga, 2012). However, another study has challenged the findings and the existence of this neutrophil subset but offered no *in vivo* functional experiments (Nagelkerke, 2014).

### *Neutrophil interaction with the microbiome*

The influence of the microbiome on the immune system is becoming increasingly clear. Granulopoiesis and neutrophil homeostasis is regulated by the mucosal commensal flora and prolonged antibiotic treatment leads to reduced neutrophil numbers. Germ free mice are neutropenic (Deshmukh, 2014; Kanther, 2014; Ohkubo, 1990). Microbiota-derived PAMPS activate PRRs, leading to G-CSF upregulation and increased neutrophil production (Balmer, 2014; Bugl, 2013). Fermentation of dietary fibres through microbiota produces short chain fatty acids (SCFA). SCFAs signal to neutrophils through chemoattractant receptors such as the G protein-

coupled receptor 43 (GPR43), regulating the resolution of inflammatory processes. Deficient SCFA-GPR43 interaction leads to exacerbated inflammation in models of asthma, colitis and arthritis in mice (Maslowski, 2009). A recent study showed that the microbial PAMPS LPS and peptidoglycan drive neutrophil ageing. Aged neutrophils ($CD62L^{lo}$) have a more activated phenotype with increased pro-inflammatory cytokine release and integrin activation. Furthermore, increased NETosis in $CD62L^{lo}$ neutrophils is associated with tissue inflammation. Antibiotic treatment reduces the number of circulating aged neutrophils and improves pathogenesis of inflammatory organ damage. Therefore, the microbiome is thought to regulate inflammatory neutrophil subsets (Zhang, 2015).

### 1.1.3 Neutrophil defects

Mutations in neutrophil-associated genes and the subsequent impairments in neutrophil function often lead to increased susceptibility and severe infection, emphasising the particular importance of neutrophils in antimicrobial defence.

In SCN granulopoiesis is blocked at the promyelocyte-myelocyte stage, leading to deficiency in mature neutrophils. SCN patients are susceptible to recurrent infections. Life-long treatment with G-CSF allows for the development of a low number of neutrophils and limited restoration of immune protection. SCN is caused by mutations in different genes, but mutations in the gene encoding for NE (ELANE) are most frequent. An accumulation of misfolded NE activates the unfolded protein response (UPR) and ER stress, promoting neutrophil apoptosis (Dale, 2000; Grenda, 2007; Kollner, 2006). Depending on the mutated gene, additional syndromes such as lymphopenia, monocytopenia, mental retardation, seizures, uropathy or albinism can accompany SCN (Kruger, 2015).

LADs are caused by autosomal recessive mutations in genes coding for selectins and integrins. Due to these defects neutrophils cannot leave the

circulation and patients have an increased susceptibility to infection (Harris, 2013). Mutations in the CD18 gene lead to lack of β2-chain integrins (αLβ2, αMβ2, αXβ2, αDβ2) in LAD-I. LAD-II is caused by mutations in the golgi guanosine diphosphate-fucose transporter (GFTP) resulting in milder infections than LAD-I. Mutation of the gene for the β-integrin adaptor kindlin-3 leads to platelet defects and subsequent bleedings and osteopetrosis-like bone defects in LAD-III.

LAD-I neutrophil recruitment defects lead to inflammatory phenotypes such as periodontal bone loss with high levels of IL-17. Efferocytosis normally downregulates the production of IL-17, underscoring the important anti-inflammatory role of neutrophils in homeostasis (Moutsopoulos, 2014).

CGD is caused by mutations in genes encoding for NADPH oxidase subunits. Mainly $gp91^{phox}$ is affected, even though mutation in any subunit can cause CGD (Segal, 2000; Stasia and Li, 2008). CGD pathology has an early onset and is defined by high morbidity and mortality. Patients have a high frequency of infections. The increased susceptibility to fungal infections, such as *A. fumigatus*, has been attributed to deficiency in NET release in CGD. Gene therapy, restoring NADPH oxidase function and NET release leads to clearance of infection (Bianchi, 2009; Rohm, 2014). Phagosomal killing is rescued upon treatment with PEGylated D-amino acid oxidase (Nakamura, 2012) or GO-containing liposomes, restoring $H_2O_2$ and HOCl production, respectively (Gerber, 2001). Unresolved infections and increased inflammation lead to a higher incidence of autoimmune disease in CGD patients.

Specific-granule deficiency is a rare disease caused by the lack of secondary and tertiary granule proteins due to deficiency in cEBPε (transcriptional regulator of granulopoiesis) (Gombart, 2001). Patients are susceptible to recurrent bacterial infections.

The warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis (WHIM) syndrome is caused by a mutation in CXCR4 leading to an increased retention of neutrophils in the BM. WHIM is therefore associated with neutropenia but also lymphopenia and monocytopenia (Hernandez, 2003).

## *1.2 NETosis*

The discovery of neutrophil extracellular traps (NETs) revolutionised the field of innate and particularly neutrophil immunity, since programmed cell death as means of antimicrobial defence had not been known before. More importantly, NETosis complemented the neutrophil antimicrobial arsenal by an extracellular defence strategy and challenged the understanding of neutrophils as simple effector cells. However, it was unknown whether neutrophil antimicrobial strategies were employed selectively and whether NET release was regulated.

### 1.2.1 NET release mechanism

When NETs were discovered, NETosis was described as a form of cell death, where nuclear chromatin decondensed and was released from the dying neutrophil in web-like structures that captured and killed microbes (Brinkmann, 2004) (**Figure I-4**). To date, this is still the most accepted and widely researched form of NETosis and the results in this thesis are based on it. However, recent evidence suggests the presence of alternative forms of NETosis such as rapid extrusion of chromatin, leaving behind live anuclear cytoplasts that continue to crawl and phagocytose (Pilsczek, 2010) (**Figure I-4**). Furthermore, release of mitochondria-derived NETs was described recently (Yousefi, 2009).

#### *Slow lytic cell death*

The main form of NETosis is caused by a cell death programme that leads to release of web-like chromatin structures into the extracellular space. Upon microbe recognition ROS are produced, the nuclear membranes disintegrate and the nucleus starts to lose its characteristic lobular shape. The chromatin decondenses into the cytoplasm. In this process neutrophil antimicrobial proteins bind to the chromatin, forming crucial NET components. The decondensed chromatin fills the entire cell generating increasing pressure,

which is released when the plasma membrane ruptures and the chromatin suddenly expands into the extracellular space (**Figure I-4**). Microbes in the direct vicinity of this event are trapped in these webs (Brinkmann, 2004). Various NET components are thought to mediate anti-microbial effects and contribute to killing of the microbes. See also **NETs in immune defence** on page 58.

Chromatin decondensation is driven by the serine protease neutrophil elastase (NE), which translocates to the nucleus where it cleaves the histones that hold the DNA in a tightly packed state (Papayannopoulos, 2010) (**Figure I-5**). Normally, NE is localised in azurophilic granules together with other proteins such as cathepsin G (CG), azurocidin (AZU) and myeloperoxidase (MPO) in a complex called azurosome (Metzler, 2014). Upon ROS production, $H_2O_2$ triggers the dissociation of NE from the complex into the cytosol.

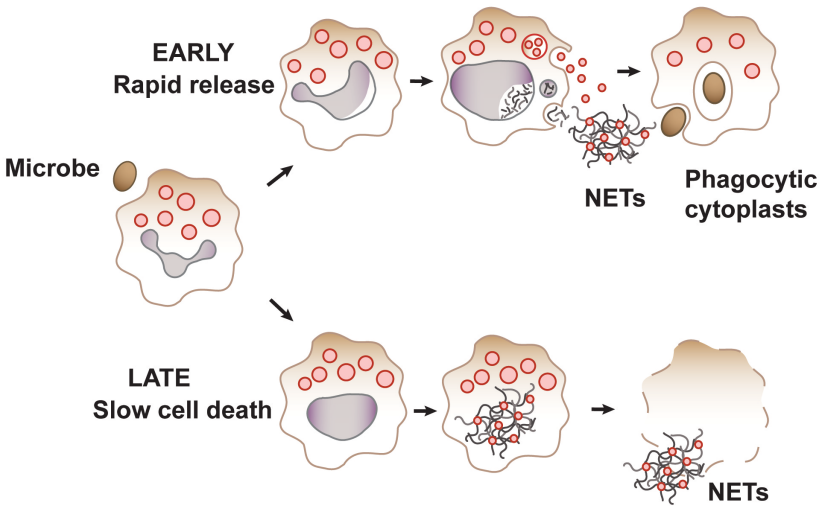


**Figure I-4 | NET release mechanisms.** Two main NET release mechanisms are known: Early rapid NET release (top row) occurs upon stimulation with *S. aureus*. The nuclear membranes separate, vesicles filled with chromatin merge with the intact plasma membrane, chromatin is released into the extracellular space. Live, anuclear cytoplasts remain and continue to phagocytose microbes. Late slow NETosis (bottom row) is a form of programmed cell death. The nuclear membranes disintegrate, the nucleus loses its lobular shape, chromatin decondenses into the cytoplasm, the plasma membrane ruptures and decondensed chromatin is released into the extracellular space. | Modified after (Branzk and Papayannopoulos, 2013), Seminars in Immunopathology.

Interestingly, this process is independent of membrane fusion that normally allows delivery of granule proteins. $H_2O_2$ activates NE's proteolytic activity in an MPO-dependent manner. The role of MPO in this process is not fully understood. However, MPO-deficient neutrophils fail to translocate and activate NE. Most likely, the enzymatic activity of MPO is not required though, since blocking MPO activity with 4-aminobenzoic acid hydrazide (ABAH) does not influence the activation process. NE is present in the cytoplasm transiently for a time of about 60 minutes and translocates to the nucleus thereafter. In the cytoplasm, activated NE binds to the actin cytoskeleton and degrades filamentous (F)-actin. This process arrests the cytoskeleton and immobilises the neutrophils at the point of stimulation, allowing for a local deployment of the NETs onto the target. Thereafter, NE dissociates from the actin and is released gradually into the nucleus (Metzler, 2014). By 120 minutes the majority of NE is found in the nucleus, where it cleaves histones thereby initiating chromatin relaxation. Subsequently, MPO follows into the nucleus, further decondensing the chromatin (**Figure I-5**). The entire process is relatively slow and takes 4 to 6 hours *in vitro* (Fuchs, 2007; Papayannopoulos, 2010).

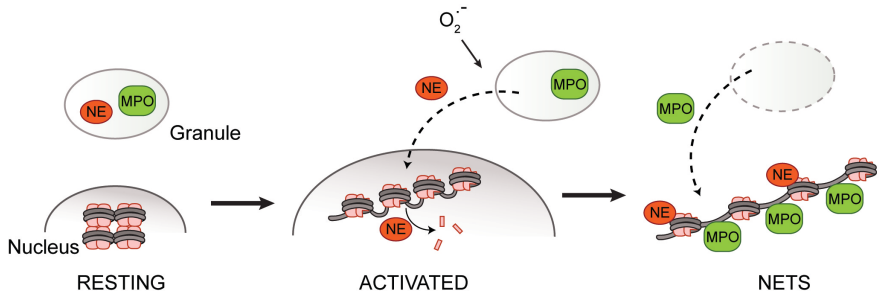


**Figure I-5 | Molecular mechanism of NET release.** In the resting neutrophil, Neutrophil elastase (NE) and myeloperoxidase (MPO) are stored in the azurophilic granules and the chromatin is tightly packed in the nucleus. Upon activation of the neutrophil through microbial stimulation, reactive oxygen species are produced. This induces translocation of NE into the nucleus, where it cleaves histones, leading to chromatin decondensation. Subsequently, MPO follows into the nucleus and contributes to complete chromatin decondensation. | Modified after (Papayannopoulos, 2010), The Journal of Cell Biology.

The receptors that trigger NETosis are unidentified. Different receptors are involved in the recognition of distinct microbes, but to date it is unknown whether these receptors are crucial for NET release. Most importantly, it is not understood at which point activation of these different receptors converges into a common NETosis pathway.

Recognition of microbes through pattern recognition receptors triggers ROS production in neutrophils. Possibly, initiation of a strong ROS burst provides a strong signal to induce NETosis. It has been shown that extrinsic glucose oxidase (GO) or its product $H_2O_2$ are sufficient to trigger NET release *in vitro* (Fuchs, 2007; Metzler, 2014). These findings support the model of $H_2O_2$-dependent NE translocation (Metzler, 2014). However, different reports describe ROS-independent NET release and it is emerging that the requirement for ROS is dependent on the microbial stimulus (Parker, 2012b).

The addition of posttranslational histone modifications alters chromatin structure and density and is therefore thought to be important for NET release. Citrullination of histone 3 is used as a clinical marker for NETosis, since its occurrence coincides reliably with NET release (Muller and Radic, 2015). However, it is still debated whether histone citrullination is required for NETosis since various studies have yielded mixed results. Peptidylarginine deiminase (PAD) 4 is an enzyme that converts the amino acid arginine into citrulline by replacing the ketimin group with a ketone group, leading to citrullination of histone 3 (Hagiwara, 2002). Inhibition of PAD4 leads to decreased histone citrullination. However, it is debated whether decreased citrullination affects NET release. Whereas some groups claim a direct link between the two phenomena (Leshner, 2012; Wang, 2009), other authors only state that they coincide (Neeli, 2008). Overexpression of PAD4 in HL-60 neutrophil-like cell lines yielded increased NET release in various studies (Leshner, 2012; Neeli, 2008; Wang, 2009). However, HL-60 cells are poor NET producers and the changes obtained in this system are not conclusive. More importantly, data show that abrogation of histone citrullination in neutrophils does not impair NET release *in vitro*

(Neeli, 2008; Warnatsch, 2015). However, others reported that neutrophils from PAD4-deficient mice are less efficient in NET release and subsequent elimination of *Shigella flexneri in vivo* (Li, 2010).

Finally, several studies have linked calcium influx in neutrophils to NET release. Calcium ionophores such as ionomycin are sufficient to trigger NETosis. The mechanism which links increased calcium flux to the NETosis pathway is not completely understood. A study suggests that the activation of calcium channels by ionomycin stimulate protein kinase C (PKC) via phospholipase C (PLC), triggering NADPH oxidase activation and production of ROS (Gupta, 2014). However, PLC activation can also recruit intracellular calcium stores, which activate calcineurin. The calcineurin pathway recruits the nuclear factor NF-ATc to the nucleus where it induces gene transcription (Gupta, 2014). Therefore, both extracellular and intracellular calcium pools are activated and used in activating NETosis. Calcium signalling after stimulation with ionomycin induces histone citrullination via PAD4. However, PMA that activates PKCα, an isotype of classical PKC, suppresses this ionomycin-induced citrullination. The inhibitory function of PKCα on histone citrullination suggests that the presence of elevated calcium levels in the cell is not an inducer of NETosis per se (Neeli and Radic, 2013).

### *Live NET extrusion*

Apart from the classical cell death-dependent NETosis pathway other NET release mechanisms have been proposed, such as live NETing neutrophils that continue to crawl and phagocytose after the release of NET material. This novel form of live NET release in response to *S. aureus* occurs rapidly within 5 to 60 minutes upon stimulation and is independent of ROS production (Pilsczek, 2010). Interestingly, no breach of the cell membrane is seen and the decondensed chromatin is released in budding vesicles. These vesicles are released into the extracellular space where they rupture and release the NET material. The remaining anuclear cytoplasts continue to crawl and scavenge the environment for phagocytosible material. NETosing

neutrophils with diffuse nuclei are highly motile and phagocytose bacteria. Anuclear neutrophils that have already excreted their entire nucleic acid material crawl slowly and continue to digest microbes. Live NET extrusion only involves a small fraction of neutrophils. At later stages other neutrophils proceed into the classical form of lytic NETosis. Live NET release has also been described *in vivo* in a model of *S. aureus* infection (Yipp, 2012). Live NET extrusion is an interesting concept since it seems to close the time window between the recognition of microbes and the late onset of NETosis at 4 hours. However, experimental verification by other groups is needed.

### *Mitochondrial NETs*

Mitochondrial DNA is released in form of extracellular traps in a fast, live NETosis mechanism. This mechanism was first discovered in eosinophils where it is dependent on priming with IL-5 or IFN-γ and stimulation with LPS or complement factor C5a (Yousefi, 2008). Later it was also found in neutrophils after GM-CSF priming and stimulation with LPS or C5a (Yousefi, 2009). Mitochondrial NETs are released rapidly within 5 minutes upon stimulation whereby the actual release happens as fast as in 1 second. The authors claim that this form of NETosis is independent of cell death since no caspase activation is found. However, even though classical NETosis is a form of cell death, it is clearly distinct from apoptosis or necrosis and no caspase activation can be found either. Therefore, further analysis of the viability of cells during mitochondrial NETosis is required. The mitochondrial origin of the DNA was confirmed through the presence of specific mitochondrial genes. Mitochondrial NETs were also found *in vivo* in patients with Crohn's disease or bacterial gastrointestinal infections. Crohn's patients have elevated levels of IL-5 and IFN-γ, which primes eosinophils accordingly (Yousefi, 2008). Furthermore, mitochondrial DNA that is released during trauma triggers classical NETosis in neutrophils. This mechanism is independent of ROS and mediated by TLR9 (Itagaki, 2015). This finding is interesting with respect to mitochondrial NETosis, since it raises the

question whether NETotic mitochondrial DNA release triggers classical nuclear NETosis, amplifying the overall response.

Generally it is not understood whether the NET mechanisms described above exist next to each other and are triggered in a timely manner in different neutrophil populations or whether different ligands trigger different forms of NETosis.

### 1.2.2 NET clearance

NET release exposes a broad range of antimicrobial effectors as well as self-antigens linked to inflammatory and autoimmune diseases. Therefore, the timely clearance of NETs is crucial for host protection. Contrary to apoptotic cells, NETs do not display eat-me signals (Kaplan and Radic, 2012). In contrast, NETs are degraded extracellularly by DNase I (Hakkim, 2010). The complement component C1q that is involved in the clearance of apoptotic cells, acts synergistically with DNase I to process NETs (Farrera and Fadeel, 2013; Gaipl, 2004). *In vitro* opsonisation by C1q mediates uptake of NETs into monocyte-derived macrophages via endocytosis. Via the phagosome NETs are shuttled to the lysosome, where they are degraded. Macrophage-driven degradation of NETs does not induce production of pro-inflammatory cytokines and represents a silent way of disposal (Farrera and Fadeel, 2013). Furthermore, NE that is present on the NETs continues to cleave histones and decondense chromatin (Papayannopoulos, 2011). In the context of cystic fibrosis this has been suggested to improve sputum solubility of patients. NETs form a component of the viscous sputum and are more easily accessible for degradation by DNase after NE-mediated decondensation.

Impaired clearance of NETs leads to increased incidence of autoimmune disease such as systemic lupus erythematosus (SLE) (Hakkim, 2010; Leffler, 2012; Mahajan, 2016). Over 60% of SLE patients are so called non-degraders since they lack the capacity to digest NETs by DNase I. This is either caused by presence of DNase inhibitors or anti-DNA antibodies that protect the DNA

from degradation by DNase (Hakkim, 2010). Other studies describe the presence of inhibitory anti-DNase antibodies in the serum of SLE patients (Yeh, 2003). Furthermore, defects in complement components such as C1q, C3 or C4 are linked to SLE. SLE patients with complement deficiencies have a lowered ability to degrade NETs (Botto, 1998; Leffler, 2012).

### 1.2.3 NETs in immune defence

Neutrophils are the first cells to arrive at the site of infection, where they encounter a broad variety of different microbes. Many fungi, bacteria, viruses and parasites are thought to trigger the release of NETs (**Table I-1**). However, it is unknown what the activating ligands are and whether NETs are released selectively upon recognition of distinct microbes.

#### *Microbial NET induction*

Possibly, different signalling pathways, receptors and ligands are involved in triggering NETosis, acting in complementary ways. Currently it is unknown at which point these different triggers converge into a common NET inducing signal. However, some conserved NET induction strategies may exist.

Recent reports state that bacterial toxins trigger NETosis by inducing necrosis via pore formation in neutrophils. The *Mycobacterium tuberculosis* protein ESAT-6 triggers neutrophil necrosis by inducing intracellular $Ca^{2+}$ overload. Necrotic neutrophils subsequently release NET material measured as free MPO and DNA (Francis, 2014). Other reports also indicate the implication of ruptured neutrophil membrane in triggering NETosis. Membrane pores induced by the *Staphylococcus aureus* pore forming toxin leukotoxin GH (lukGH) or electroporation leads to increased NETosis (Malachowa, 2013). A similar effect is induced by the *Mannheimia haemolytica* leukotoxin (LKT) (Aulik, 2010). However, pore formation could simply cause nuclear and granular material to leak into the extracellular space, making it difficult to distinguish between NET release and necrosis by

| | Species/ Agent | References |
|---|---|---|
| **Bacteria** | *Burkholderia pseudomallei* | (Riyapa, 2012) |
| | *Echerichia coli* | (Grinberg, 2008; Lippolis, 2006; McDonald, 2012; Munafo, 2009) |
| | *Eimeria bovis* | (Behrendt, 2010; Munoz-Caro, 2015b) |
| | *Enterococcus faecalis* | (Lippolis, 2006) |
| | *Haemophilus influenzae* | (Hong, 2009; Juneau, 2011) |
| | *Helicobacter pylori* | (Hakkim, 2011) |
| | *Klebsiella pneumoniae* | (Papayannopoulos, 2010; Sorensen, 2014) |
| | *Lactococcus lactis* | (Buchanan, 2006) |
| | *Leptospira spp* | (Scharrig, 2015) |
| | *Listeria monocytogenes* | (Ermert, 2009; Munafo, 2009) |
| | *Mannheimia haemolytica* | (Aulik, 2010) |
| | *Mycobacterium tuberculosis* | (Francis, 2014; Ramos-Kichik, 2009) |
| | *Neisseria ghonorrhoeae* | (Gunderson and Seifert, 2015; Juneau, 2015b) |
| | *Porphyromonas gingivalis* | (Jayaprakash, 2015) |
| | *Pseudomonas aeruginosa* | (Kamoshida, 2015; Yoo, 2014) |
| | *Pseudomonas fluorescens* | (Chi and Sun, 2016) |
| | *Shigella flexneri* | (Brinkmann, 2004; Li, 2010) |
| | *Staphylococcus aureus* | (Berends, 2010; Fuchs, 2007; Malachowa, 2013; Pilsczek, 2010; Yipp, 2012) |
| | *Streptococcus pyogenes* | (Buchanan, 2006; Lauth, 2009; Sumby, 2005) |
| | *Streptococcus suis* | (Zhao, 2015) |
| | *Yersinia enterolitica* | (Casutt-Meyer, 2010) |
| | *Yersinia pseudotuberculosis* | (Gillenius and Urban, 2015) |
| | *Lipopolysaccharide (LPS)* | (Yousefi, 2008) |
| **Fungi** | *Aspergillus fumigatus* | (Bruns, 2010; Loures, 2015; McCormick, 2010; Rohm, 2014) |
| | *Aspergillus nidulans* | (Bianchi, 2009; Bianchi, 2011) |
| | *Candida albicans* | (Byrd, 2013; Byrd, 2015; Ermert, 2009; Metzler, 2011; Papayannopoulos, 2010; Urban, 2009; Urban, 2006) |
| | *Candida glabrata* | (Springer, 2010) |
| | *Cryptococcus gattii* | (Springer, 2010) |
| | *Cryptococcus neoformans* | (Urban, 2009) |
| | *Paracoccidioides brasiliensis* | (Mejia, 2015) |
| | *Paracoccidioides spp* | (Bachiega, 2016; Della Coletta, 2015) |

**Table I-1 | NET-inducing microbes.**

| | | |
|---|---|---|
| **Parasites** | *Cryptosporidium parvum* | (Munoz-Caro, 2015a) |
| | *Eimeria bovis* | (Behrendt, 2010) |
| | *Leishmania amazonensis (amastigotes)* | (Guimaraes-Costa, 2009) |
| | *Leishmania amazonensis, L. major, L. chagasi (promastigotes)* | (Guimaraes-Costa, 2009; Wardini, 2010) |
| | *Leishmania donovani* | (Gabriel, 2010) |
| | *Leishmania mexicana* | (Hurrell, 2015) |
| | *Plasmodium falciparum* | (Baker, 2008) |
| | *Strongyloides stercoralis* | (Bonne-Annee, 2014) |
| | *Toxoplasma gondii* | (Abi Abdallah, 2012) |
| **Viruses** | *HIV-1* | (Saitoh, 2012) |
| | *Influenza* | (Hemmers, 2011; Narasaraju, 2011) |
| | *Rabbit poxvirus (MYXV)* | (Jenne, 2013) |
| | *Respiratory syncytial Virus (RSV)* | (Funchal, 2015) |

**Table I-1 | NET-inducing microbes (continued).**

solely measuring the release of NET material. Furthermore, a mechanistic explanation of how pore formation and necrosis would induce NETosis is still missing. Further research into this phenomenon is required.

Though ROS products can induce NETosis directly (Akong-Moore, 2012), it is unknown whether a strong microbial ROS induction is sufficient for NET release in the absence of additional signals. The conidia form of the fungus *Paracoccidioides brasiliensis* induce NETosis independent of ROS production, whereas the yeast form depends on NADPH activity for NET release. Interestingly, a mutant *P. brasiliensis* strain with reduced expression of the alternative oxidase (AOX), important for reduction of ROS, induced higher levels of NETs (Mejia, 2015). *Yersinia pseudotuberculosis* triggers ROS production and NET release in neutrophils via the adhesive protein invasin. *E. coli* expressing the *Y. pseudotuberculosis* invasin gain the ability to induce NETs and show increased ROS production (Gillenius and Urban, 2015).

## *NET antimicrobial mechanisms*

NETs are an antimicrobial strategy with the aim to limit infection. Originally NETs were thought to entrap microbes to physically contain dissemination. A more targeted delivery of antimicrobials through NETs could contribute to the antimicrobial activity of NETs (Brinkmann, 2004). Whether NETs kill the entrapped microbes is debated and differs between microbes. Microbes have developed several NET evasion strategies. See **Microbial NET evasion** on page 64.

Already in the initial description NETs were suggested to prevent microbial dissemination by ensnaring and physically trapping microbes (Brinkmann, 2004). Experimental bacterial sepsis with *E. coli* induces NETosis, increasing bacterial trapping and reducing dissemination *in vivo* (McDonald, 2012). NETs from myeloid related protein (MRP) 14-deficient neutrophils fail to control *Klebsiella pneumoniae in vitro*. In MRP14 knockout mice *K. pneumoniae* disseminates more readily, which support the role of NETs as effectors against bacterial dissemination (Achouiti, 2012).

Another interesting concept is the immobilisation of microbes to facilitate attack by other immune cells. The helminth *Strongyloides stercoralis* induces NETs. Even though NETs are ineffective in killing *S. stercoralis* larvae, the entrapment by NETs is required for killing by macrophages and other incoming neutrophils (Bonne-Annee, 2014).

Contact of NETs with bacteria can lead to growth inhibition of the bacteria and potentially lower the infectivity upon reinfection with these bacteria *in vivo*. *Pseudomonas fluorescens* triggers NET release in fish neutrophils. Trapped *P. fluorescens* are inhibited in replication. Re-infection of fish with these trapped bacteria results in lowered dissemination and colonization indicating an anti-bacterial effect (Chi and Sun, 2016). A similar effect is seen on the parasite *Eimeria bovis*, which induces NETs and is trapped and immobilized by these. Infection of bovine umbilical vein endothelial cell

(BUVEC) cultures *in vitro* was lowered with trapped *E. bovis* sporozoites as compared to controls (Behrendt, 2010). These studies indicate NET-associated damage to trapped microbes that result in decreased infectivity beyond prevention of physical dissemination. However, it is unknown how NETs bind to microbes and research is required into which components of the microbes are altered by this contact, causing the decreased infectivity.

The ultimate aim of each antimicrobial strategy is elimination of the invading pathogen. However, the killing capacity of NETs is variable and seems to be dependent on the entrapped pathogen. *Mannheimia haemolytica* and *Leptospira* species trigger NET release and are trapped and subsequently killed in those (Aulik, 2010; Scharrig, 2015). *E. coli* is killed by shrimp haemocyte ETs (Ng, 2013). Interestingly, NETs induced by *Neisseria ghonorrhoeae* are inefficient in killing these gonococci but do kill the common vaginal bacterium *Lactobacillus crispatus* (Gunderson and Seifert, 2015). Microbes have developed escape strategies such as polysaccharide capsules that prevent killing via NETs. *Streptococcus suis* capsule negative mutants are killed in NETs (Zhao, 2015). Furthermore, NETs are ineffective in killing some parasites such as *Strongyloides stercoralis*, *Leishmania mexicana*, *Eimeria bovis* (Behrendt, 2010; Bonne-Annee, 2014; Hurrell, 2015). However, promastigotes of *Leishmania amozonensis*, *L. major*, *L. chagasi* and amastigotes of *L. amazonensis* are efficiently killed by NETosis (Guimaraes-Costa, 2009).

The mechanisms by which NETs kill microbes are not fully understood but different mechanism are proposed. Possibly a combination of different killing mechanism may be involved. NETs contain wide range of antimicrobials that are known to kill. The ion chelator calprotectin is found on NETs and scavenges zinc and manganese from the environment, depriving fungi from essential growth conditions. While some studies report calprotectin-dependent killing of NET-trapped fungi such as *Aspergillus nidulans* (Bianchi, 2011) other studies propose growth-inhibition without final killing (Clark, 2015). *In vivo* studies in *C. albicans* infection models only provide indirect

evidence for calprotectin-dependent killing by NETs. Calprotectin-deficient mice succumb to pulmonary candidiasis and spreading of subcutaneous *C. albicans* infection is increased as compared to control animals (Urban, 2009; Urban, 2006). However, it is not clear whether this is directly dependent on NET-mediated killing.

Furthermore, the antimicrobial peptide LL-37 has been shown to play a role in NET-mediated killing. Neutralisation of LL-37 by group A *Streptococcus* (GAS) serotype M1T1 via the surface protein M1 leads to impaired neutrophil- and NET-mediated killing (LaRock, 2015; Lauth, 2009). M1-deficient GAS mutants are efficiently killed by NETs.

Interestingly, NETs can also associate with microbial components that enhance the anti-microbial activity of the extracellular traps. *Staphylococcus epidermidis* produces the phenol-soluble modulin δ-toxin (PSMγ) that associates with the NET component cathelicidin. δ-toxin-containing NETs show increased killing of GAS (Cogen, 2010).

The importance of ROS for NET-mediated killing is debated. However, a study shows killing of *Staphylococcus aureus* dependent on HOCl. The authors describe a mechanism by which MPO bound to NETs may metabolise $H_2O_2$ to HOCl. In this *in vitro* study, addition of exogenous $H_2O_2$ was required for *S. aureus* killing. (Parker, 2012a). *In vivo* the infiltration of activated neutrophils may provide a source of $H_2O_2$. However, ROS mediated microbial killing may not necessarily be NET-mediated. Evidence suggests that ROS is required for fungal killing. Direct neutrophil-fungal contact triggers a ROS burst and degranulation, independent of NET formation (Diamond and Clark, 1982; Jahn, 1996; Levitz and Diamond, 1985; Schaffner, 1986).

There are certain technical caveats with NET killing assays used in some studies. The major problem may be clumping of microbes that are trapped in (pre-formed) NETs followed by plating of these “colony forming units” on agar plates. The resulting colony counts may underestimate the numbers of

actually alive microbes. Treatment of the trapped microbes with DNase to dissolve the NETs before plating could solve this issue. Interestingly, where DNase digestion was used the observed killing is comparatively low (Bruns, 2010; Menegazzi, 2012; Parker, 2012a).

### *Microbial NET evasion*

Triggering NETosis combined with the inefficiency of the NETs to kill the entrapped microbes is an immune evasion strategy, harming the host. NET release that does not lead to efficient microbe elimination, depletes the neutrophil pool, further weakening the immune response (Gunderson and Seifert, 2015). Furthermore, continued NET triggering which is not haltered by microbial clearance increases the risk for host damage due to the tissue toxicity of NETs.

Microbes have developed several NET evasion strategies, which will be discussed below.

Polysaccharide capsules are a common virulence strategy in bacteria, which allow the microbe to prevent immune strategies such as uptake by phagocytes. However, more recent reports indicate that capsules can also prevent killing by NETosis or NET induction in the first place. The bacterium *Streptococcus suis* contains a polysaccharide capsule that prevents both phagocytosis and NET killing (Zhao, 2015). The *Streptococcus pneumoniae* polysaccharide capsule reduces trapping in and killing by NETs with increased susceptibility in capsule deficient mutants (Wartha, 2007). *Burkholderia pseudomallei* induces NETs dependent on the capsular polysaccharide I (CPS-I). CPS-I-deficient *B. pseudomallei* mutants show a decreased capacity to induce NETosis (Riyapa, 2012).

*Haemophilus influenzae* forms biofilms and bacterial-induced NETs are a component of these biofilms. *H. influenzae* is resistant to NET killing dependent on the expression of catalase (hktE) and peroxiredoxin-

glutaredoxin (pdgX). Strains lacking these enzymes show increased susceptibility to peroxide as well as decreased survival within NETs (Juneau, 2015a).

*Streptococcus pyogenes* (group A *Streptococcus*, GAS) M1 serotype induces more NETs, but is more resistant to NET killing as compared to serotypes not expressing the M1 protein. M1 sequesters the active forms of cathelicidin LL-37 (human) and mCRAMP (mouse) leading to impaired NET killing (LaRock, 2015; Lauth, 2009).

Nontypeable *Haemophilus influenzae* (NTHI) is characterised by formation of biofilm composed of a polymeric lattice that contains lipooligosaccharides (LOS). Interestingly, NETs have been identified as host-derived component in these biofilms. NTHI survive within NETs and resist phagocytosis dependent on the expression of LOS. Mutants with altered LOS composition and structure are susceptible to NET-mediated killing (Hong, 2009; Juneau, 2011). However, it is unclear, what the contribution of these biofilm components to NET evasion is compared to expression of ROS reducing enzymes as described by the same group. (See above (Juneau, 2015a).)

The most common NET evasion strategy is the degradation of NETs by microbe-derived nucleases. *Neisseria gonhorroea* induces the release of NETs and similarly their degradation over time, dependent on the putatively secreted thermonuclease (Nuc) that is normally required for biofilm remodelling. Since NETs have antimicrobial activity against *N. gonhorroea*, Nuc expression enhances bacterial survival (Juneau, 2015b). *Streptococcus pyogenes* (group A *Streptococcus*, GAS) M1 serotype expresses the extracellular DNase Sda1. GAS eliminate NETs by Sda1 digestion and are more resistant to neutrophil killing than an Sda1 KO mutant, which also induce smaller lesions and lower bacterial titres *in vivo* than wild type GAS (Buchanan, 2006). GAS deficient for three extracellular DNases (NAK) induce smaller lesions and no viable bacteria in a mouse infection model (Sumby, 2005). *Streptococcus suis* induces and degrades NETs *in vitro* via the secreted

nuclease A (SsnA). A SsnA mutant is attenuated in NET degradation and is less protected against NET antimicrobial activity *in vitro* (de Buhr, 2014). Another *S. suis* nuclease Endonuclease A (EndAsuis) degrades NETs dependent on $Mg^{+}$. However, contrary to SsnA EndAsuis deficiency did not lead to higher susceptibility to NET killing (de Buhr, 2015). *Staphylococcus aureus* nuclease (nuc) degrades NETs. Nuc deficient strains are more susceptible to extracellular killing. Therefore infection with Nuc-sufficient wild type *S. aureus* leads to delayed clearance in the lung and increased mortality in mice as compared to infection with a Nuc deficient mutant (Berends, 2010). Similarly, a *Streptococcus pneumonia* endonuclease (endA)-deficient strain is less virulent and mice show increased survival (Beiter, 2006).

Microbes are also able to directly alter neutrophil responses including NET release by inducing signalling cascades via either direct cell interaction or provision of signalling mediators. The *S. aureus* enzymes nuclease (nuc) and adenosine synthase (adsA) convert NETs to deoxyadenosine (dAdo), a deoxyribonucleoside base of DNA. dAdo causes accumulation of intracellular dATP, which subsequently quenches DNA synthesis and triggers caspase-3-dependent immune cell death, excluding macrophages from *S. aureus* abscesses (Thammavongsa, 2013). Therefore, *S. aureus* does not only cause NET degradation, but also utilizes the degradation products for further immune suppression. Group B *Streptococcus* expresses a sialylated polysaccharide in its capsule, which engages Siglec-9 on neutrophils. Siglec-9 signalling dampens neutrophil reactivity characterised by a reduced ROS burst and decreased NET release (Carlin, 2009).

Interestingly, NET evasion is a primarily bacterial phenomenon. This might mainly be explained by the fact that most pathogenic bacteria express nucleases, whereas fungi are nuclease free.

## *NET interaction with the gut microbiota*

The influence of the microbiome on the immune system has become increasingly clear in the last few years. Therefore, it is not surprising that gut bacteria also influence neutrophil immunity, including NETosis. The gut contains a plethora of bacteria representing potential NET triggers. Unregulated NETosis would lead to severe bowel inflammation, due to the tissue toxicity of NETs.

Interestingly, bacterial cultures isolated from *Citrobacter rodentium*-infected mice enriched for probiotic *Lactobacillus* species did not induce NET release *in vitro* in contrast to cultures enriched for aerobic and facultative anaerobic bacteria or *E. coli*. Furthermore, *Lactobacillus rhamnosus* suppresses NET release upon stimulation with *S. aureus* and PMA *in vitro*. *S. aureus*- mediated cytotoxicity was reduced in the presence of *L. rhamnosus*, which the authors attribute to reduced *S. aureus*-induced NETosis (Vong, 2014; Vong, 2015). Microbiota and microbiome-derived components (LPS, peptidoglycan) drive neutrophil ageing *in vivo*. Aged neutrophils show an activated phenotype, which includes increased NET formation. Antibiotic treatment decreases neutrophil ageing and reduces NET release followed by decreased tissue damage in mice (Zhang, 2015). Therefore, increased NET induction due to dysbiosis could contribute to inflammatory bowel conditions.

## *NETs and adaptive immunity*

Various interactions of neutrophils with the adaptive immune system are known. However, research is needed into the implication of NETs in these interactions. A recent report describes a B cell-helper subset of neutrophils ($N_{BH}$) in the marginal zone (MZ) of the spleen, characterised by an activated phenotype, that drives class switching, somatic hypermutation and immunoglobulin production in B cells. $N_{BH}$ constitutively release NETs in close proximity to MZ B cells. The authors of the study suggest that NETs released by $N_{BH}$ enhance T-independent immunoglobulin B cell responses by

trapping antigen and more directly by delivering immunostimulatory DNA (Puga, 2012). These findings indicate a protective function of NETs by initializing the adoptive immune system, by priming of B cells through constitutively released NETs.

### *NET deficiencies*

There are several lines of evidence that suggest that NETosis is crucial for antimicrobial defence (Bianchi, 2009; Bianchi, 2011). However, the overall contribution of NETs in immunity is not clearly defined.

ROS play a crucial role in triggering NETosis for most stimuli (Fuchs, 2007). However, since ROS are also implicated in other neutrophil immune functions it is difficult to dissect the role of NETs in ROS deficient patients completely. Patients with ROS deficiencies suffer from severe infections with pathogenic and commensal microbes. Interestingly however, these patients are specifically prone to fungal diseases (Bianchi, 2009; Parker and Winterbourn, 2012; Segal, 2000; Stasia and Li, 2008). The NADPH oxidase drives metabolism of oxygen to superoxide, which is then further metabolised into hydrogen peroxide ($H_2O_2$) and hypochlorite. NADPH oxidase deficiency leads to chronic granulomatous disease (CGD). CGD patients suffer from severe and recurrent opportunistic infections with mostly fungi such as *Aspergillus fumigatus* (Antachopoulos, 2010). CGD neutrophils are unable to form NETs upon stimulation with PMA or bacteria and addition of $H_2O_2$ restores NET formation (Fuchs, 2007). In humans, gene therapy rescued NADPH oxidase activity and restored antifungal immunity against *A. fumigatus*. Patients neutrophils treated with gene therapy killed *A. fumigatus* hyphae mostly by NETosis (Bianchi, 2009; Bianchi, 2011) In mice, deficiency of the NADPH oxidase subunit $p47^{phox}$ abrogates NET release. *A. fumigatus* infections are not resolved in these mice (Rohm, 2014). Similarly, CGD mice deficient in the gp91 subunit of the NADPH oxidase are deficient in NET release upon infection with *C. albicans* (Ermert, 2009).

Myeloperoxidase is another important enzyme in the ROS cascade metabolising $H_2O_2$ into hypochlorite. Patients with MPO mutations show varied, complex clinical manifestation since many of these mutations only cause partial loss of enzyme activity (Kameoka, 2004; Kutter, 1998; Kutter, 2000; Metzler, 2011). However, complete MPO deficiency leads to chronic fungal susceptibility. Interestingly, residual MPO activity of only 3% is sufficient for NETosis. However, completely MPO-deficient neutrophils are unable to release NETs (Metzler, 2011; Parker, 2012b). The only sporadic episodes of fungal infection in MPO-deficient patients are explained by slightly impaired but overall sufficient capacity of their neutrophils to phagocytose, which allows them to eliminate most infections in the early stages.

Patients with Papillon-Lefèvre syndrome (PLS) have a genetic mutation in cysteine protease cathepsin C (CTSC) that is required for processing of serine proteases, leading to deficiency in cathepsin G, proteinase 3 (PR3) and NE. A recent study shows that PLS neutrophils do not release NETs *in vitro*. Interestingly, the studied patient did not show any signs of immunodeficiency apart from periodontal complications. Furthermore, patient neutrophils were sufficient in bacterial killing when challenged with *S. aureus* or *Klebsiella pneumoniae* (Sorensen, 2014).

The requirement for PAD4 and histone citrullination in NETosis is debated. However, several studies indicate that PAD4 deficiency abrogates NETosis. *In vitro*, neutrophils from PAD4-deficient mice are impaired in NET-mediated killing of *Shigella flexneri* and group A *streptococcus* (GAS). NET deficiency impacts mice in a model of GAS-induced necrotizing fasciitis where PAD4-deficient mice develop bigger lesions compared to WT controls (Li, 2010). Contrarily, PAD4-dependent NETosis is dispensable in an influenza A infection model, where PAD4-deficient mice show similar virus titres, viral replication and survival rates compared to control animals (Hemmers, 2011). These findings indicate that even though PAD4 deficiency seems to impair NET release, the importance of this impairment is dependent on the

microbial stimulus. Furthermore, no human PAD4 deficiencies are known. In a study of polymicrobial sepsis, where PAD4-mediated NET release did not affect bacteremia in mice, the authors concluded that PAD4 inhibition in inflammatory diseases would not likely increase host vulnerability to infection in humans (Martinod, 2015).

### 1.2.4 NETs in disease

NETs are not only antimicrobial, but excessive NETosis and impaired NET clearance are involved in a number of autoimmune and inflammatory diseases. Free double-stranded DNA acts as DAMP and induces activation of immune responses. Extracellular histones are cytotoxic and can cause tissue inflammation.

#### *Autoimmunity*

NETs are a major source of extracellular chromatin, neutrophil proteins and antimicrobial molecules and have therefore been implicated in the onset of autoimmune diseases.

SLE patients display high titres of anti-neutrophil cytoplasmic antibodies (ANCA) and anti-nuclear antibodies (ANA) (Tsokos, 2011), linking NETosis to this disease. The NET-associated antimicrobial peptide LL-37 enhances the ability of DNA to activate plasmacytoid dendritic cells (pDCs) via TLR9 (Garcia-Romo, 2011; Lande, 2011). Interestingly, a subset of so-called low-density granulocytes undergoes spontaneous NETosis (Denny, 2010; Villanueva, 2011), which induces pDCs to secrete IFN$\alpha$. IFN$\alpha$ promotes auto-reactivity (Garcia-Romo, 2011; Villanueva, 2011) and primes neutrophils to respond to immune complexes with NET release. This process further activates pDCs (Garcia-Romo, 2011; Lande, 2011) and represents a vicious cycle of inflammation. Accordingly, impaired NET clearance in SLE patients is associated with increased autoantibody titres. DNase inhibitors and anti-NET antibodies that protect from DNase activity (Hakkim, 2010) as well as anti-

DNase antibodies (Yeh, 2003) have been identified as causes for impaired NET degradation.

Rheumatoid arthritis (RA) is a systemic disease that causes chronic inflammation. High levels of citrullinated proteins and anti-citrullinated peptide antibodies (ACPA) are disease indicators (Chang, 2005) and NETs are present in the synovial fluids, rheumatoid nodules and in the skin of RA patients. Patient synovial fluids and ACPA-containing serum induce NETs in healthy neutrophils (Khandpur, 2013). NETs contain auto antigens, which feed into a cycle of continued NET release. Furthermore, metalloprotease-8 (MMP-8) is associated with RA NETs (Khandpur, 2013) and is critical for tissue destruction in arthritis (Malemud, 2006).

### *Inflammation*

Gout is the most common form of arthritis. It is caused by precipitation of uric acid in sodium containing fluids, yielding monosodium urate (MSU) crystals. MSU crystals activate the NALP3 inflammasome and induce IL-1β release. The subsequent production of IL-8 leads to recruitment of neutrophils. Interestingly, MSU crystals induce NET release, which cause persisting inflammation (Mitroulis, 2011).

The lack of sputum clearance leads to chronic airway inflammation in CF. It has been shown that high levels of decondensed extracellular DNA contribute to sputum viscosity. Accordingly, DNase treatment improves sputum solubilisation in CF patients (Cantin, 1998; Vogelmeier and Döring, 1996). NETs are an important source of this extracellular DNA. Other NET components such as MPO and NE have also been found (Marcos, 2010; Papayannopoulos, 2011; Ratjen, 2008). However, NE that remains bound to chromatin and continues to degrade histones, further decondensing the chromatin and allowing for a better accessibility to therapeutic DNases (Papayannopoulos, 2011). NE inhibitors block the effectiveness of DNase therapy.

NETs interact with endothelial cells, platelets, coagulation factors and red blood cells (von Bruhl, 2012), providing scaffolds in the circulation that promote thrombus formation (Gould, 2015). Accordingly, neutrophil depletion or intravenous DNase injection prevents thrombus formation (Brill, 2012; von Bruhl, 2012). IL-8 and ROS produced by activated endothelial cells leads to recruitment of additional neutrophils and release of NETs (Gupta, 2010). Furthermore, endothelial cells release fibrin and von Willebrand factor (vWF), which promote blood coagulation and formation of thrombus scaffolds. vWF has a high affinity for histones and binds to NETs (Gonias, 1985; Ward, 1997). Additionally, histones have been shown to inhibit anti-coagulants in the plasma (Ammollo, 2011). Finally, NETs interact with platelets (Gould, 2014; Renesto and Chignard, 1993; Si-Tahar, 1997) and SLE patients with anti-platelet antibodies have an increased risk for thrombosis (Al-Homood, 2012).

Atherosclerosis is a disease that is characterised by the deposition of fatty plaques in the arteries. Cathelicidin-related antimicrobial peptide (CRAMP), the murine homologue of LL-37) recruits inflammatory monocytes to the arteries. NETs are implicated in the formation of atherosclerosis. Complexes of CRAMP and DNA stimulate pDCs in the fatty plaques to generate autoantibodies, which aggravates the course of atherosclerosis (Doring, 2012a; Doring, 2012b). Double-stranded DNA, nucleosomes and MPO-DNA complexes are increased in the plasma of patients with severe coronary atherosclerosis (Borissoff, 2013). Recent data from our group shows that cholesterol crystals trigger NETosis. The NETs are able to prime macrophages for the release of IL-1β. This activates Th17 cells, which further amplify immune cell recruitment to the plaques (Nahrendorf and Swirski, 2015; Warnatsch, 2015).

NETs are released spontaneously and PMA-dependent NETosis is increased under conditions of high glucose *in vitro* (Menegazzo, 2015). Interestingly, a study shows spontaneous NETosis in diabetes, whereas LPS-induced NETosis is impaired at high glucose concentrations. The authors conclude that a

chronic pro-inflammatory state induces constitutive NET release, causing a weak response upon stimulation (Joshi, 2013). NET formation is impaired in diabetic patients during infection with *Burkholderia pseudomallei*, causing a high susceptibility to this bacterium (Riyapa, 2012). However, diabetic patients show increased levels of NET components such as NE and DNA in the plasma in absence of infection (Menegazzo, 2015). Furthermore, the excess NETosis has been shown to impair wound healing in diabetic patients (Wong, 2015). NETosis is also linked to type I diabetes, as high amounts of NET enzymes correlate with autoantibodies against the insulin-releasing β-cells (Leslie and Bradford, 2014; Wang, 2014).

### *Cancer*

Neutrophils play a major role in cancer as they are part of the inflammatory infiltrate in tumours. However, their specific function is debated since both pro- and anti-tumour effects have been described (Brandau, 2013; Gregory and Houghton, 2011). NETs were found in Lewis lung carcinoma (Ho-Tin-Noe, 2009) and Ewing sarcoma (Berger-Achituv, 2013). Furthermore, a tumorigenic role of NE was attributed in lung adenocarcinoma, where tumours grow slower in NE-deficient mice (Houghton, 2010). Finally, NETs promote cancer metastasis. During systemic infection NETs are deposited on the blood vessels and trap circulating cancer cells. These cells proliferate and form cancerous nodules. Therefore, NETs promote tumour progression (Cools-Lartigue, 2013; Cools-Lartigue, 2014).

## 1.3 Anti-fungal immunity

Fungi are heterotroph eukaryotes that are ubiquitous in the environment. Yeast and filamentous growth forms exist. Fungi sense their surroundings and respond to environmental cues that promote their survival through specific reprogramming (Brown, 2007; Hube, 2009). Interaction with plants, animals and humans can be symbiotic, commensal, latent or pathogenic. The high incidence of fungi in the environment causes continuous exposure. Humans inhale hundreds of spores and small yeast daily.

Fungi cause a wide spectrum of diseases, which range from cutaneous lesions and acute self-limiting pulmonary disease in immunocompetent people to inflammatory diseases and life-threatening infections. Even non-lethal, skin and mucosal infections are difficult to treat and often recurrent. Over one million life threatening infections are caused by fungi each year reaching up to 95% mortality. Fungal infections are often difficult to diagnose and drug resistance and the lack of new antifungal drugs and vaccines poses a big health problem (Brown, 2012a). Most fungi are opportunistic and the incidence of systemic infections has increased due to the use of immunosuppressive drugs, the raise of acquired immunodeficiencies (e.g. HIV) as well as the increase in clinical interventions in intensive care (Pappas, 2010).

Immunity to fungal infections is extremely dependent on the site of infection and the fungal species. Fungi have co-evolved with the host over time and host immune mechanisms antagonise with fungal strategies. Host defence is complex and requires both the innate and adaptive immune system. The initial recognition by epithelial cells and macrophages induces pro-inflammatory responses and initiates immune cell activation. A barrier breach induces antigen presentation and adaptive immune cell recruitment.

### 1.3.1 *Candida albicans*

*C. albicans* is a diploid fungus that is a commensal in around 30-70% of the human population. It colonises the intestinal and vaginal mucosa as well as the skin. In healthy individuals it rarely causes disease symptoms (Perlroth, 2007). However, *C. albicans* is an opportunistic pathogen that can cause severe infections when host immunity is disturbed. Invasive candidiasis occurs in patients with neutropenia, pancreatitis or renal insufficiency and after treatment with glucocorticosteroids or systemic antibiotics. Further risk factors are indwelling devices such as catheters, parenteral nutrition or major surgery. Systemic candidiasis is difficult to treat with current antifungal drugs. Improved diagnostics and new drug targets are required (Brown, 2012b; Gudlaugsson, 2003). *C. albicans* is not naturally a commensal in mice, which makes it an ideal model to study the involvement of both innate and adaptive immunity in antifungal defence.

*C. albicans* is a dimorphic fungus that grows as unicellular budding yeast or filamentous hyphae (**Figure I-6a-c**). Furthermore, it also forms pseudohyphae, which are chains of elongated yeast cells that are not separating from the mother cell after budding. Therefore, *C. albicans* is also called polymorphic. Whereas other fungal species have environmental reservoirs, *C. albicans* yeast and hyphae grow uniquely inside mammalian host tissues (Romani, 2003).

*C. albicans* is covered by a cell wall that contains mainly carbohydrates and cell wall proteins that are foreign to the host. This allows for a good discrimination between self and non-self by the immune system. Most of the fungal PAMPs are cell wall-related (Netea, 2015a; Romani, 2011). The *C. albicans* cell wall is composed of two layers: The outer layer is made up of glycoproteins. About 80% of the covalently linked sugars are O- and N-linked mannans. The outer layer is attached to the inner layer by glycosylphosphatidylinositol (GPI) residues that are linked to β-glucans (Cutler, 2001; Ernst and Prill, 2001). The inner layer contains the structural

polysaccharides chitin and β-glucan that provide strength and shape to the cells (**Figure I-6d**). The dry weight of a yeast particle contains about 2% chitin, 40% β-1,3-glucan and 20% β-1,6-glucan (Netea, 2008). The composition of the cell wall can change for example in situations where β-glucan synthesis is disturbed. Hyphae contain 3 to 5 times more chitin as compared to yeast cells (Munro, 1998). The β-glucan content is similar, but is shielded in hyphae by the mannan layer. It is mainly exposed in yeast bud scars (Gantner, 2005; Marakalala, 2013).

The change of morphology from yeast to hyphae is associated with changes in the cell wall composition and architecture. Many genes that encode hypha-specific proteins are upregulated during morphoswitching. The morphogenesis of *C. albicans* can be induced by different mechanisms, triggered by different environmental factors. Most commonly hyphal growth is induced via the cyclic AMP-protein kinase A complex (PKA) (cAMP-PKA) signalling pathway. Many environmental cues regulate this pathway and

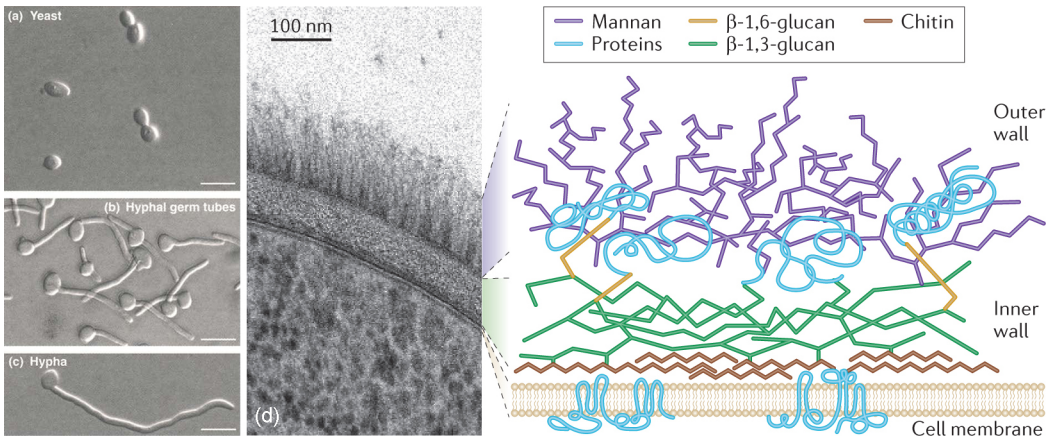


**Figure I-6 | *C. albicans* morphology and cell wall composition.** (**a** - **c**) *C. albicans* morphologies: budding yeast particles (a), germinating *C. albicans* hyphae (b) and fully developed hyphae (c). (**d**) *C. albicans* cell wall composition. The cell wall consists of two distinct layers that are connected via glycosylphosphatidylinositol (GPI) residues that are linked to β-1,6-glucan. The outer layer contains glycoproteins that are mannosylated via O- and N-linkages. The inner layer consists of the structural polysaccharides chitin and mannan.| (a-c) Reprinted from Trends in Microbiology (Sudbery, 2004), Copyright 2004, with permission from Elsevier. Modified. (d) Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Gow, 2012), copyright 2012.

most are integrated by adenyl cyclase Cyr1 (also Cdc35). Cyr1 can be activated directly by $CO_2$ or serum muramyl dipeptide (MDP), the minimal peptidoglycan structure that is present on gram-positive bacteria (Hall, 2010; Xu, 2008). Glucose and amino acids signal via Cyr1 indirectly through small GTPases (Maidan, 2005). Other factors activate the cAMP-PKA pathway via unidentified modes: Temperatures below 35°C stop hyphal germination via activation of the heat shock protein Hsp90 (Shapiro, 2009). Furthermore, accumulation of the quorum sensing molecule farnesol in situations of high fungal density downregulates cAMP-PKA signalling (Deveau, 2010). Activation of Cyr1 leads to increased levels of cAMP, which induce the dissociation of the PKA regulatory subunit (Bcy1) from the catalytic subunits (Tpk1 and Tpk2), leading to activation of PKA. PKA phosphorylates and activates the transcription factor Efg1, which induces hypha-specific genes (Bockmuhl and Ernst, 2001; Sohn, 2003). These include adhesins, invasins, immune modulators and the cyclin Hgc1. Hgc1 interacts with cell division cycle 28 (Cdc28), which mediates the activation of the septins Cdc11 and Sep7, driving polarised growth and cell separation (Bishop, 2010). Hgc-deficiency leads to a yeast-locked state (Zheng, 2004).

Immunity to *C. albicans* differs dependent on the colonisation or invasion state. Colonising yeast particles are mainly commensal whereas hyphal germination triggers invasion and activation of an antifungal immune cascade.

### 1.3.2 Innate anti-fungal immunity

The skin and mucosal surfaces are the first line of contact with microbes, including fungi. Epithelial and endothelial cells form a physical barrier that protects the underlying tissues. Many fungi are commensal in healthy individuals and are kept in check by the innate immune system. When this immune balance is disturbed or the epithelial barrier is broken colonising commensals can become invasive. Innate immune cells posses a set of broad specificity pattern recognition receptors (PRR) that recognise pathogen

associated molecular patterns (PAMPs). Fungal PAMPs are mainly cell wall derived and include β-glucan, chitin and mannans. Different fungal species express different PAMPs on their surface that vary dependent on the morphotype, growth stage and fungal environment (Bourgeois, 2010; van de Veerdonk, 2008). Innate immune cells involved in antifungal defence include monocytes, macrophages, neutrophils, NK cells and some innate-like cells such as innate lymphoid cells (ILCs). Engagement of PRRs triggers downstream activation of signalling cascades leading to phagocytosis, release of antimicrobial effectors, cytokine production and subsequent recruitment of other effector cell types as well as activation of adaptive immunity. A functioning innate immune response is able to inhibit fungal growth or directly trigger fungal killing.

### *Innate effector cells*

A complex interplay of innate immune cells is required for sufficient antifungal defence. Each effector cell type plays specific roles in the recognition of fungi and the deployment of antifungal strategies (**Figure I-7**).

Epithelial and endothelial cells contribute to immune defence by forming a physical barrier between microbes and the host tissue. ECs provide the first point of contact with the host immune system. They express TLRs and can upregulate CLRs as seen during pulmonary aspergillosis (Guo and Wu, 2009; Sun, 2012; Weindl, 2010). ECs produce pro-inflammatory cytokines and IL-8 upon TLR activation or stimulation with antimicrobials (Guo and Wu, 2009; Wagener, 2013). Morphological changes of *C. albicans* from yeast to hyphal form induces penetration of ECs and leads to tissue invasion. Two main ways of invasion are known: *C. albicans* triggers TLR signalling and activation of NF-κB and JUN (AP-1) independent of the fungal morphology. This leads to induced endocytosis by the ECs and transgression through the epithelial barrier (Weindl, 2007). However, *C. albicans* can also invade by active penetration. Morphoswitching to hyphae activates MAPK1 and FOS signalling in ECs (Moyes, 2011; Moyes, 2010). It is not known which *C. albicans* cell wall

components or ECs receptors are required for this interaction. The initiated signalling causes the release of pro-inflammatory cytokines from ECs and initiation of a downstream host response. IL-22 released by Th17 T cells or ILCs triggers production of β-defensins in ECs, which controls the commensal state of *C. albicans* (Eyerich, 2011; Tomalka, 2015).

Neutrophils are the first cells that are recruited to the site of infection by chemokines such as CXCL8 (IL-8) secreted by ECs and tissue resident macrophages (Netea, 2008). Neutrophils are the only host cells that can fully prevent the morphoswitch of *C. albicans* and neutropenia is an important risk factor for invasive fungal infections (Brown, 2011; Uzun, 2001). Neutrophils exert a variety of antifungal defence strategies. ROS production, phagocytosis and release of NETs are dependent on NADPH oxidase and MPO. Non-oxidative killing strategies include the release of antimicrobial factors such as lysozyme, lactoferrin, elastase, β-defensins, gelatinase, cathepsin G, calprotectin, azurocidin and cathelicidin. Cathelicidin is processed by PR3 into the antimicrobial peptide LL-37, which causes disruption of fungal membranes, inhibition of biofilm formation, fungal adhesion, chemotaxis, ROS production and inhibition of neutrophil apoptosis (Alalwani, 2010; Tsai, 2011). Mice deficient in calprotectin are more susceptible to fungal infection (Urban, 2009). See also **Neutrophil immune responses** on page 31.

Monocytes are blood-borne and migrate into the tissues upon inflammatory signals where they differentiate into macrophages. Mice deficient in the chemokine receptor CXCR1 (IL8RA) fail to recruit monocytes to the tissues and suffer from renal failure upon infection with *C. albicans* (Lionakis, 2013). Decreased CXCR1 function in humans leads to increases susceptibility to disseminated candidiasis (Lionakis, 2013). Similarly, failure to recruit blood monocytes by CCL2 binding to CCR2 leads to increased incidents of invasive candidiasis (Ngo, 2014). In the tissues macrophages acquire distinct phenotypes. Classical activation by inflammatory cytokines such as IFN-γ triggers M1 macrophages that acquire an antimicrobial phenotype and release pro-inflammatory IL-12. Alternatively activated M2 macrophages

receive anti-inflammatory signals like TGF-β and mediate tissue repair via secretion of IL-10 (Mosser and Edwards, 2008). Macrophage phenotypes are relevant in antifungal defence since it has been shown that M2 macrophages confer susceptibility in pulmonary *Cryptococcus neoformans* infection. However, immunisation with a vaccine strain induces M1 macrophages, promoting rapid resolution of infection with wild-type *C. neoformans* (Hardison, 2012). Plasticity between M1 and M2 macrophages is important against infections with *Paracoccidioides* (Feriotti, 2013). Interestingly, in *C. albicans* infection depletion of tissue resident M2 macrophages leads to increased susceptibility (Lionakis, 2013; Qian, 1994). Importantly, monocytes and macrophages confer trained immunity, a short-term innate immune memory. Monocytes stimulated with β-glucan acquire a protective phenotype upon re-stimulation. This innate memory is caused by epigenetic reprogramming and metabolic changes (Cheng, 2014; Quintin, 2012; Saeed, 2014).

NK cells are normally known for their role in antiviral and antitumor defence. But NK cells also have antifungal properties as evidenced in infections with *C. albicans, A. fumigatus, C. neoformans* and *Pneumocystis* species (Islam, 2013; Kelly, 2013; Longhi, 2012; Schmidt, 2013b). NK cells kill yeast particles via perforins, secrete inflammatory cytokines and kill infected host cells. However, the role of NK cells in antifungal immunity is complex and NK cells also influence other antifungal immune cells (Schmidt, 2013b). Therefore depletion of NK cells has been shown to have varying effects ranging from no effect to increased susceptibility to disseminated candidiasis (Romani, 1993; Whitney, 2014). Depletion of NK cells in lymphocyte deficient SCID mice increases invasive candidiasis but does not affect WT mice (Quintin, 2014). Additionally, some fungal species, such as *Rhizopus oryzae* have evolved to actively suppress NK cell function (Schmidt, 2013a).

Dendritic cells are classically involved in antiviral immunity. Even though they are less efficient in phagocytosing and killing fungi, they have been implicated in antifungal immunity (Netea, 2004). They are activated via the

Syk/IRF5 pathway and respond by producing IFN-β (del Fresno, 2013). Most importantly however, DCs process and present antigen to induce an adaptive T cell response. It is not fully understood which DC subsets are required for antifungal immunity even though evidence suggests that mice deficient for plasmacytoid DCs (pDCs) are susceptible to invasive aspergillosis (Ramirez-Ortiz, 2011). A recent study suggests that in a model of cutaneous candidiasis, nocireceptive pathways in the skin drive production of IL-23 by CD301b$^+$ dermal DCs. IL-23 signalling stimulates IL-17 production in γδ T cells, leading to protective immunity (Kashem, 2015b).

The involvement of other innate immune cells in antifungal immunity is less investigated and in most parts indicated by indirect evidence.

As indicated above, γδ T cells are suspected to play a role in antifungal immunity due to their capacity to produce large amounts of IL-17 (Gladiator, 2013). γδ T cells express an invariant T cell receptor (TCR) containing a γ and a δ chain that doesn't require recombination and therefore classifies these cells as innate-like. γδ T cells are mainly found in the gut mucosa.

Innate lymphoid cells (ILCs) are found in mucosal tissues. By their use of lineage-defining transcription factors and the production of hallmark cytokines ILCs are divided in 3 groups that mirror the compartments of T helper cells. ILC1s produce IFN-γ, ILC2s produce IL-4 and IL-13, whereas ILC3s release IL-17 (Spits, 2013). ILCs are a relatively new field and their involvement in antifungal defence is partially contradicting and likely to be dependent on the fungal species and site of infection. ILC3s are thought to be important in mucosal candidiasis since Rag-1-deficient mice depleted of IL-17 secreting ILCs fail to control mucosal candidiasis (Gladiator, 2013). It is unclear how ILCs contribute to immunity during invasive fungal infections.

Innate NK (iNK) T cells have an invariant TCR that recognises lipid antigen in the context of an MHC CD1d complex. CD1d-deficient mice that lack iNK T cells have an increased susceptibility to *A. fumigatus* (Cohen, 2011).

In mice *C. albicans* in the blood stream bind and activate platelets, which have antimicrobial effects through induction of CCL5 (RANTES) and platelet factor 4. Platelet-rich plasma inhibits the growth of *C. albicans* (Drago, 2013; Robert, 2000).

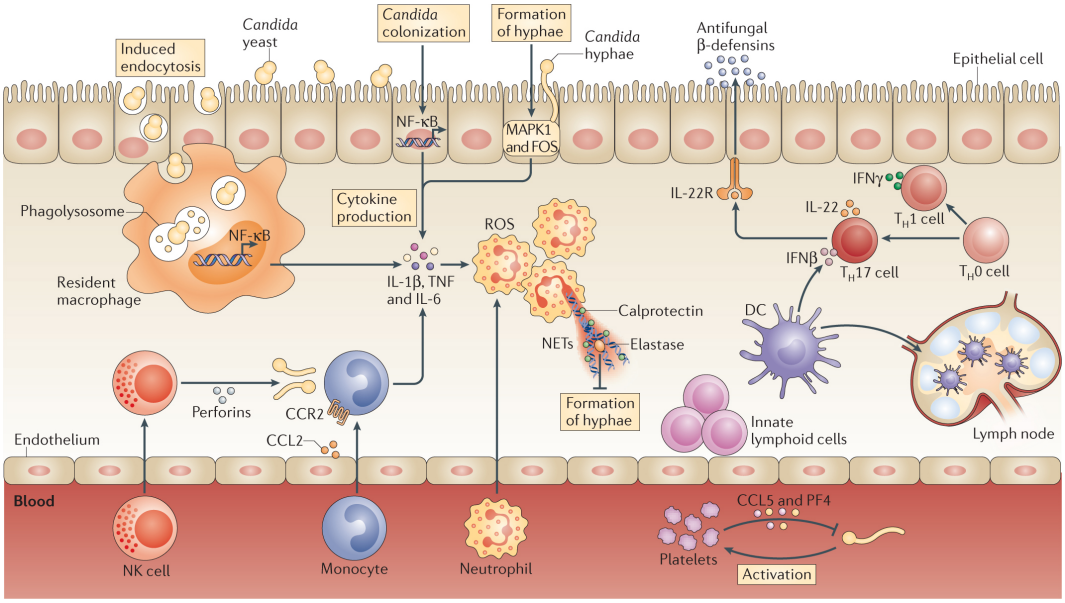


**Figure I-7 | Antifungal effector cells.** Epithelial cells (ECs) provide a physical barrier between fungal particles and host tissues. They express pattern recognition receptors and produce inflammatory cytokines that lead to the recruitment of phagocytic cells. Upon stimulation by T cell-derived IL-22 ECs produce antifungal β-defensins. Fungi such as *Candida albicans* can breach the tissue barrier by induced endocytosis or active penetration upon hyphal germination. Once in the tissue fungi are countered by tissue-resident macrophages that phagocytose the particles and release inflammatory mediators that recruit neutrophils. Neutrophils are the most potent antifungal cells and release reactive oxygen species (ROS), antimicrobials such as calprotectin and neutrophil extracellular traps (NETs). Dendritic cells (DCs) migrate to the lymph nodes where they modulate T helper cell responses. Th17 cells have an important antifungal function since the release of IL-17 recruits more neutrophils, whereas IL-22 stimulates ECs to produce defensins. Invasive fungi such as *C. albicans* activate platelets in the blood stream, which in turn release antifungal mediators such as CCL5 and platelet factor 4 (PF4). Natural killer (NK) cells release antifungal perforins. | Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Netea, 2015a), copyright 2015.

### 1.3.3 Adaptive anti-fungal immunity – T cell responses

In most cases the epithelial and phagocyte responses are enough to control the fungi at barrier sites and prevent dissemination. However, when the infection is not controlled immediately, adaptive immunity provides the second line of defence.

Innate immune sensing activates $CD4^+$ T helper cells. DCs are specialised in receiving fungal signals and activate different pathways that shape T cell responses accordingly (Bonifazi, 2009). The innate and adaptive antifungal responses depend on the balance of different DC subsets (Claudia, 2002). In cases of severe fungal disease or relapsing diseases inflammatory DCs develop. Inflammatory DCs initiate Th17 and Th2 cell responses via MyD88 signalling. In cases of asymptomatic or mild disease, tolerogenic DCs initiate Th1 and Treg cell differentiation via signalling through TRIF. In some cases fungi exploit balance between different T cell effector types and establish commensalism and infection (Romani, 2002).

Th1 helper cell responses correlate with protective immunity and effective fungal vaccination (Spellberg, 2008; Zhang, 2009). Th1 cells produce IFN-γ and induce the production of opsonising antibodies, which lead to optimal activation of phagocytes at the site of infection. Failure to raise these activating signals leads to persistence of fungal infection (Romani, 2004). Furthermore, a direct inhibition of Th1 cell proliferation by crytptococcal polysaccharides leads to a defective adaptive immunity and persistent *crytptococcus* infections (Yauch, 2006).

IL-4 and IL-13 production by DCs favours the production of Th2 helper T cells, dampens the Th1 response and promotes alternatively activated macrophages. Th2 responses promotes fungal infection, disease relapse and fungal allergy (Muller, 2007). Limiting IL-4 signalling restores antifungal immunity in mice (Szymczak and Deepe, 2009).

Th17 cells reside mainly in mucosal tissues. They have functions in the defence against extracellular pathogens and are involved in autoimmune and allergic disorders. However, Th17 cells are also the main T effector cell type involved in antifungal immunity. They are activated by macrophages and DCs through signalling via the mannose receptor, Syk, CARD9 and MyD88 (LeibundGut-Landmann, 2007; Robinson, 2009) and inhibited through type I IFNs. Th17 cells are found in the fungal-specific T memory cell repertoire (Acosta-Rodriguez, 2007). In *C. albicans* infections Th17 cells support a Th1 response and suppress Th2 cells (Conti, 2009). Defective IL-17 signalling leads to an increased Th2 response and fungal-associated allergy. The host defence to fungal infections vary dependent on the site of infection and whether disease is compartmentalised or disseminated (Dongari-Bagtzoglou and Fidel, 2005). Th17 cells are important for mucosal antifungal immunity and are the main producers of IL-17 in the mucosa. During oropharyngal candidiasis (OPC) a strong induction of Th17 genes in the oral mucosa takes place including CXCL2, CXCL5 and G-CSF that mobilise the neutrophil pool, antimicrobials such as β-defensin-3, S100 proteins and CLRs like mincle, dectin-2 and dectin-1 (Aujla, 2007). Saliva has candidacidal activity that is lost in Th17-deficient mice (Conti, 2009). OPC is increased in CXCR2-deficient mice due to the lack of neutrophil recruitment and mice suffer from weight loss, increased fungal burden and an in turn increased IL-17 gene signature. Depletion of neutrophils in wild-type mice has a comparable effect indicating the importance of IL-17 signalling through recruitment of antifungal neutrophils (Huppler, 2014). Similar to mice Th17 defects in humans cause increased susceptibility to mucosal but not systemic candidiasis (Hernandez-Santos and Gaffen, 2012; Liu, 2011; Puel, 2011). Psoriasis patients that are treated with anti-IL-17 antibodies show increased susceptibility to fungal infections (Langley, 2014). Apart from Th17 T cell some innate sources of IL-17 are known. NK T cells, γδ T cells, $CD4^{-}CD8^{-}TCR\beta^{+}$ cells and natural Th17 cells that do not require activation via a TCR, produce IL-17 (Cua and Tato, 2010; Spits, 2013). Neutrophils, mast cells, macrophages and paneth cells are also considered to be sources of IL-17 (Hueber, 2010; Lin, 2011; Werner,

2011). In dermal candidiasis γδ T cells are the main producers of IL-17. *C. albicans* downregulates Th17 responses and failure to do so results in chronic inflammation (Cheng, 2010).

The immune system must eliminate fungi and at the same time avoid tissue damage. In chronic fungal disease and non-resolving inflammation regulatory T cells produce high levels of IL-10 to supress production of IFN-γ (Romani and Puccetti, 2006). During fungal infection, Treg cells may shift the immune response from protection to immunosuppression (Ferreira, 2010).

### *Immunity to yeast and hyphae*

An important virulence factor of *C. albicans* is its capacity to switch morphology from small yeast particles to large hyphal filaments. Immunity towards these two fungal morphoforms differs in many aspects (Gow, 2012). *C. albicans* yeast but not hyphae induce a tolerogenic DC phenotype characterised by the production of IL-12 via signalling through TLR4 (d'Ostiani, 2000). Recognition of hyphae differs due to an altered exposure of β-glucan on the surface. β-glucan is exposed in the yeast bud scars but is shielded in hyphae by a layer of mannoproteins. However, some reports also state a slight exposure of β-glucan on hyphae due to incomplete coverage by mannan fibrils that are stretched during hyphal outgrowth (Gantner, 2005). Apart from different cytokine induction, hyphae also differ in the activation of inflammasomes, which is not triggered by the yeast particles. Differential inflammasome activation is an important discrimination mechanism between colonisation and invasion (Cheng, 2011; Gow, 2012). The chemistry of cell wall polysaccharides differs between yeast and hyphae and could be an additional means of discrimination between the colonising and invasive morphoforms forms (Lowman, 2014). Interestingly, *Candida glabrata* does not form hyphae and is yet virulent (Brunke and Hube, 2013).

## 1.4 Introduction summary

Neutrophils are important innate immune cells and their release and recruitment is tightly regulated. In the tissues they encounter a large variety of microbes. Neutrophils possess different immune strategies such as degranulation of antimicrobial agents, ROS production, phagocytosis and NET release. However, it is not understood whether neutrophils are able to regulate their antimicrobial strategies and use them selectively to target distinct microbes. Neutrophils are thought to be of particular importance for antifungal defence since neutrophil deficiencies often lead to fungal infections. However, it is unknown whether neutrophils can control fungi and whether selected antimicrobial strategies are employed. Furthermore, fungi induce NET release but it is not understood which receptors lead to initiation of the NETosis pathway. The following study will address these open questions.

# *2 RESULTS*

## 2.5 NETosis is a selective strategy against large microbes

The results discussed in this chapter were published in Nature Immunology (Branzk, 2014). Some of the fluorescent microscopy stainings shown were produced in collaboration with Dr Qian Wang and Ms Aleksandra Lubojemska. The *S. pneumoniae* infection was done by Dr Gregory T Ellis. Contributions are acknowledged in the figure legends. Some analysis was performed in collaboration with Dr Venizelos Papayannopoulos.

### 2.5.1 Background and aims

Neutrophils encounter a variety of microbes at the sites of infection. However, neutrophils have long been considered to undertake a single antimicrobial program with limited means of adaptation and it is unknown whether they can respond to different microbes selectively. However, the discovery of NETs (Fuchs, 2007) indicated that neutrophils utilize several antimicrobial strategies. NETs have been shown to be involved in antifungal defence through mechanisms that are poorly understood (Bianchi, 2009; Bianchi, 2011; Metzler, 2011). Emerging evidence shows that NETs are involved in a variety of immune pathologies (Knight, 2012; Villanueva, 2011). This raises the question whether a regulatory mechanism exists that prevents excessive NET release.

Despite recent work showing that macrophages are able to discriminate between soluble microbial ligands and particulate microbes (Goodridge, 2011), it is unknown whether immune cells discriminate between microbial particles of differing size. NETosis is an extracellular defence strategy but it is unknown whether it is specifically targeted at larger pathogens.

Therefore, we investigated the importance of NETs in immune defence and asked why NETs where potently released in response to fungal pathogens. Secondly, we explored whether neutrophils are able to discriminate between

microbes of differing size and adapt their antimicrobial strategies accordingly. Specifically, the following questions were addressed:

Can neutrophils sense the size of microbes and selectively initiate intra- or extracellular defence mechanisms?

Is NETosis down-regulated in order to avoid unnecessary exposure of NETs to the host environment in situations where other antimicrobial strategies are sufficient to counter microbes?

If yes, what is the molecular mechanism of this regulated release?

### 2.5.2 NETosis depends on microbe size

#### *Only hyphae induce NETs but not yeast*

Like many fungi, *C. albicans* is a dimorphic fungus and can be present as small budding yeast particles or large filamentous hyphae. It is a good inducer of NETosis *in vitro* in the presence of blood plasma (Ermert, 2009; Urban, 2006). Plasma is one of the factors that drives switching of the morphology in *C. albicans,* resulting in the formation of large filamentous hyphae (Nantel, 2002). Importantly, it has not previously been resolved whether NET release can be triggered by both morphoforms of *C. albicans*.

To dissect the influence of plasma on NETosis, we stimulated human peripheral neutrophils with wild type (WT) *C. albicans* in the presence of 3% blood plasma. *C. albicans* triggered NET release only in the presence of plasma. Interestingly, NETosis coincided with the formation of *C. albicans* hyphae (**Figure 1a**).

Next we tested whether plasma drives NETosis by promoting transformation from yeast to hyphae. A yeast-locked *C. albicans* strain carries a mutation in

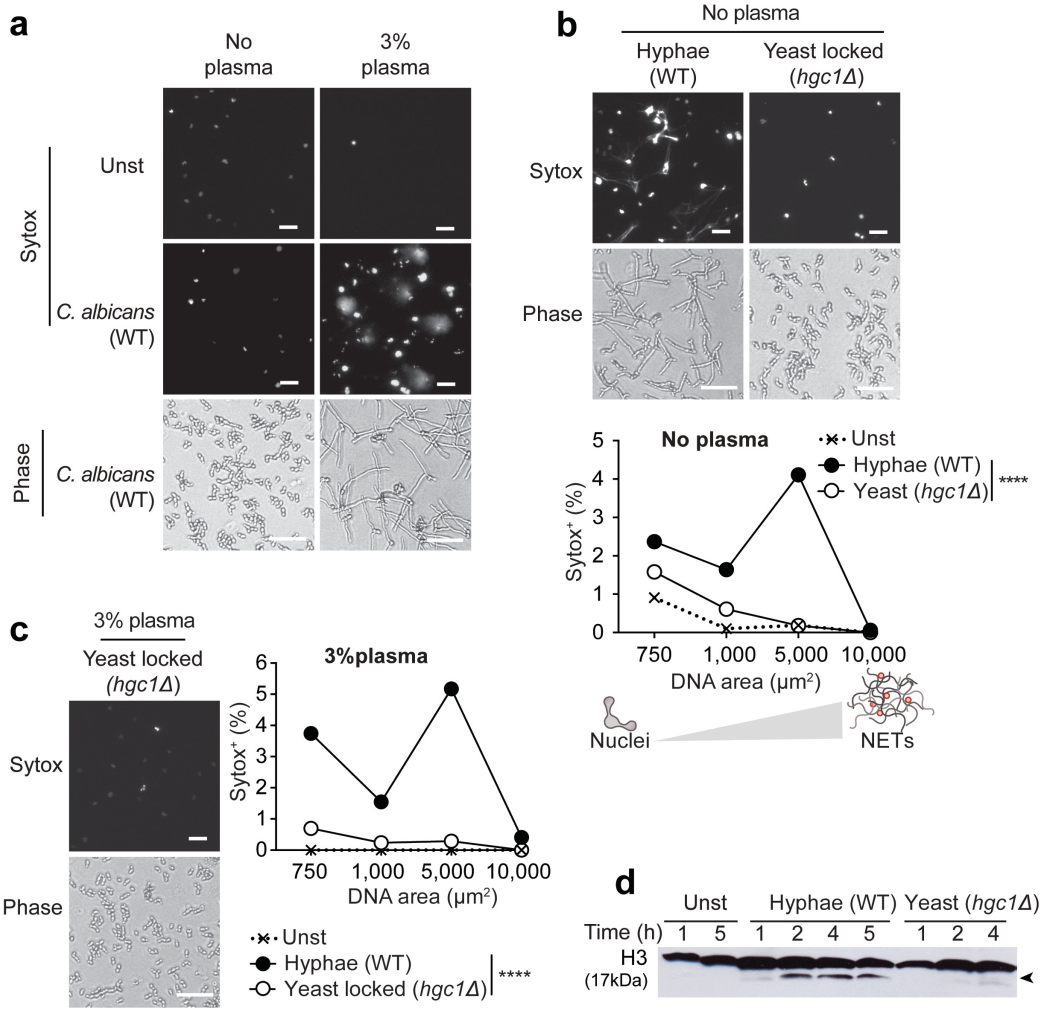


**Figure 1 | Only hyphae induce NETs but not yeast.** (**a**) Release of NETs by human peripheral neutrophils left unstimulated (Unst) or stimulated with wild-type (WT) *C. albicans* in the presence (right) or absence (left) of 3% plasma. Extracellular DNA was stained with SYTOX 4 hours after stimulation (top panels). Brightfield microscopy of the neutrophils and *C. albicans* (bottom). (**b**) Release of NETs by human peripheral neutrophils stimulated with *C. albicans* WT hyphae or *hgc1*Δ yeast (*hgc1*Δ), in the absence of plasma (top). Quantification of NET release (bottom), presented as $SYTOX^+$ events relative to total neutrophils. Below, graphic representation of $SYTOX^+$ area ranging from condensed necrotic nuclei (left) to decondensed NETotic chromatin (right). (**c**) Release of NETs by human peripheral neutrophils stimulated with *C. albicans hgc1*Δ yeast, in the presence of 3% plasma (left). Quantification of NET release by human peripheral neutrophils left unstimulated or stimulated with *C. albicans* pre-formed wild-type hyphae or *hgc1*Δ yeast in the presence of 3% plasma. (**d**) Immunoblot analysis of degradation (arrow) of histone H3 (17 kDa) in neutrophils left unstimulated or stimulated with *C. albicans* pre-formed wild-type hyphae or *hgc1*Δ yeast, for the indicated times in the absence of plasma. | Multiplicity of infection (MOI) = 10. Scale bars = 50 μm. Statistics by one-way ANOVA followed by Tukey's multiple comparison post-test. **** $p \leq 0.0001$. Data are representative of at least three independent experiments.

the *hgc1* gene that is required for hyphal formation (Zheng, 2004). We pre-cultured the WT and the *hgcΔ1* yeast-locked *C. albicans* strains in conditions that favour hyphal growth. Under these conditions only WT *C. albicans* formed hyphae, whereas the *hgc1Δ* mutant remained small yeast particles (**Figure 1b**). We then stimulated neutrophils with both strains in the absence of plasma. Interestingly, without plasma only the WT hyphae but not the small yeast form triggered the release of NETs (**Figure 1b**). Furthermore, in the presence of plasma the small yeast form was unable to trigger NET release, contrary to the WT form of the fungus (**Figure 1c**). The *hgc1Δ C. albicans* mutant remained in the small budding yeast form and did not convert to hyphae even in the presence of plasma. All following *in vitro* experiments were carried out in the presence of 3% plasma to provide optimal culture conditions for the neutrophils.

Histone degradation is a hallmark of NETosis (Papayannopoulos, 2010). Therefore, we investigated whether or not histones were degraded upon stimulation with pre-formed *C. albicans* WT hyphae and *hgc1Δ* yeast particles. We probed the degradation of histone 3 over the course of 5 hours by Western blotting. Unstimulated neutrophils showed no histone degradation, indicated by the stable presence of a 17 kDa band, corresponding to the full-length histone 3 protein. However, stimulation with *C. albicans* wild type hyphae induced histone 3 degradation already as early as 1 hour after stimulation. This is indicated by the appearance of a protein fraction smaller than 17 kDa. The degradation was fully developed by 2 hours and remained stable until 5 hours post stimulation. In contrast, stimulation with the small yeast locked *hgc1Δ* particles induced minimal histone 3 degradation at 2 hours and remained negligible up until 4 hours post stimulation (**Figure 1d**).

These results indicate that NET release is independent of the presence of plasma. However, plasma indirectly enables NETosis by driving the switch in fungal morphology from yeast to hyphae. Therefore, these results uncover

the mechanism by which plasma potentiates NETosis. Importantly, NETosis is induced by the large *C. albicans* hyphae form but not by the small yeast.

### *NET-inducing capacity of C. albicans is size-dependent*

We investigated whether the discrimination between the two *C. albicans* growth forms was based on differences in the expression of surface molecules or differences in the size of the particles.

Therefore, we devised an experimental approach, which prevented phagocytic uptake of the *C. albicans* particles while at the same time allowing for direct contact and sensing of the microbe by the neutrophils. We modified a commercially available transwell so that it could be placed directly over the neutrophils that were seeded at the bottom of the well. For stimulation we added WT *C. albicans* hyphae or small *hgc1Δ* yeast particles to the upper part of the transwell (**Figure 2a**).

We confirmed the direct contact of the neutrophils with the *C. albicans* particles by staining the bottom of the transwell filter with DAPI. This revealed that a large number of neutrophils attached to the bottom side of the filter (**Figure 2b**).

Stimulation with WT *C. albicans* hyphae in this modified transwell triggered the release of NETs to a similar extend as compared to a classical stimulation set up as described before. Surprisingly, the small *hgc1Δ* mutant *C. albicans* yeast particles that were prohibited from phagocytic uptake gained the capacity to induce release of NETs comparable to the WT hyphae (**Figure 2c** and **d**). Importantly, small *hgc1Δ C. albicans* yeast particles were not able to trigger the release of NETs in a classical suspended transwell that did not allow for direct contact with the neutrophils (**Figure 2e**).

These results show that only particles that cannot be phagocytosed by neutrophils trigger release of NETs. This applies to particles of large size or

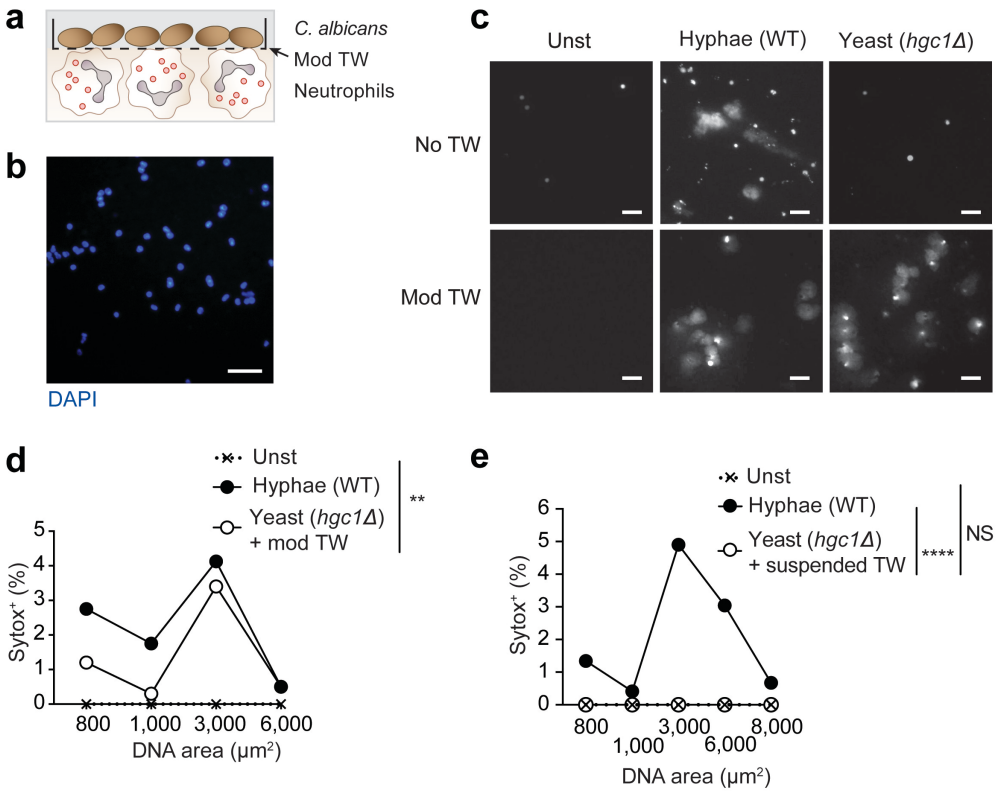


**Figure 2 | Preventing uptake of yeast induces NETosis.** (**a**) A modified transwell (Mod TW) allows contact between *C. albicans* particles (top) and neutrophils (bottom) but prevents phagocytosis. (**b**) Human peripheral neutrophils attached to the bottom of the modified transwell membrane 1 hour post stimulation. Nuclei were stained with DAPI (blue). (**c**) Release of NETs by human peripheral neutrophils left unstimulated (Unst) or stimulated with *C. albicans* wild-type (WT) hyphae or *hgc1Δ* yeast, directly (No TW, top) or with separation by a modified Transwell (Mod TW, bottom). Extracellular DNA was stained with SYTOX 4 hours after stimulation. (**d**) Quantification of NET release by human peripheral neutrophils left unstimulated or stimulated with *C. albicans* pre-formed WT hyphae directly or *hgc1Δ* yeast with separation by modified Transwell (yeast (*hgc1Δ*) + mod TW), presented as SYTOX$^+$ events relative to total neutrophils. (**e**) Quantification of NET release by human peripheral neutrophils left unstimulated or stimulated with *C. albicans* pre-formed WT hyphae directly or *hgc1Δ* yeast with separation by a suspended Transwell that did not allow direct contact but exchange of soluble factors (yeast (*hgc1Δ*) + suspended TW). | Multiplicity of infection (MOI) = 10. Scale bars = 50 µm. Statistics by one-way ANOVA followed by Tukey's multiple comparison post-test. NS (not significant) $p>0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$. Data are representative of at least three independent experiments.

when uptake is hindered by a physical barrier such as a modified transwell. Small phagocytosible particles do not induce NETosis. Furthermore, direct contact is required and no soluble mediators activate NET release in this context. To further corroborate the size-dependent concept, we tested whether fragmentation of the large hyphal filaments into small phagocytosible fragments would abrogate formation of NET release.

We fragmented *C. albicans* hyphae by high-pressure homogenisation (**Figure 3a**). We confirmed by confocal microscopy staining that the fragmented hyphae were phagocytosed by the neutrophils. In contrast, large hyphal filaments were not taken up and were located extracellularly (**Figure 3b**).

We used heat-inactivated WT *C. albicans* hyphae for fragmentation to exclude that killing of the particles altered the NET-inducing response. *C. albicans* killing by heat-inactivation did not abrogate NET release (**Figure 3c** and **d**). Heat-inactivation even increased the rate of NET release, most likely due to exposure of further activating cell wall components (**Figure 3d**). Surprisingly, small fragments of the same WT hyphae lost their capacity to induce NETs (**Figure 3e** and **f**).

Taken together, these results suggest that NET release depends on microbe size. Only large fungal particles that cannot be taken up by phagocytosis trigger NETosis, whereas small particles of the same fungal microbe are phagocytosed and do not elicit a NETosis response. Both morphoforms are equally able to trigger NET release when phagocytosis is prevented, suggesting that the response is not regulated by the differential expression of fungal surface molecules.

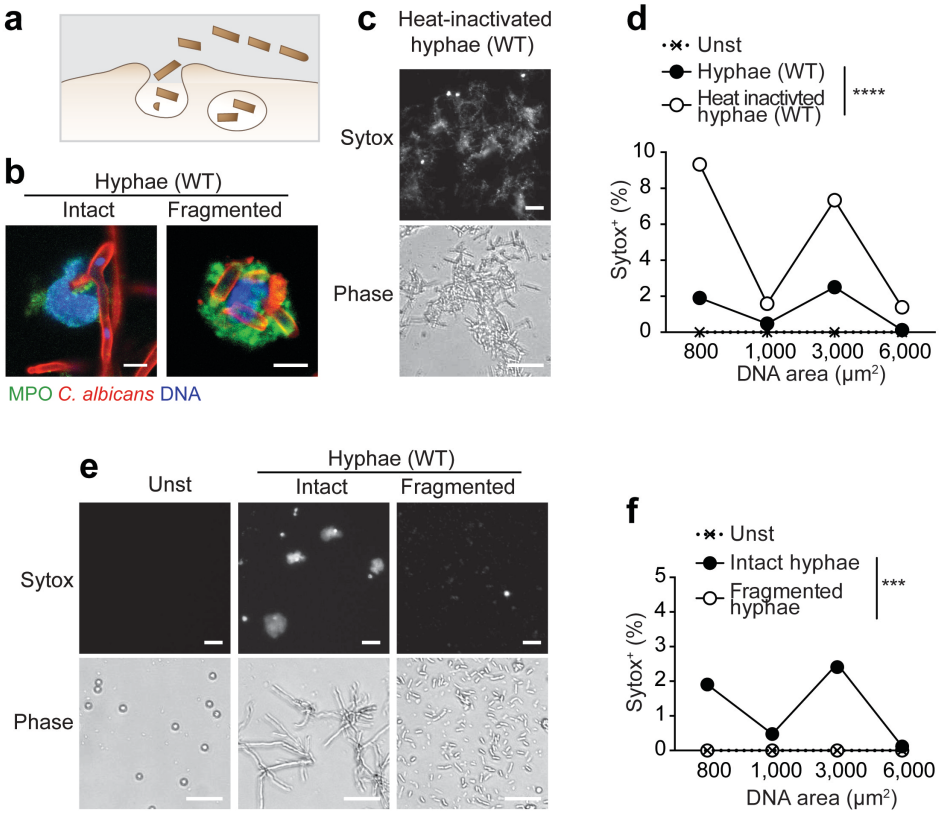


**Figure 3 | Fragmented hyphae are phagocytosed and fail to induce NETosis.** (**a**) WT *C. albicans* hyphae were heat-inactivated and fragmented by pressure. The hyphal fragments were small enough to be phagocytosed. (**b**) Confocal microscopy of human peripheral neutrophils stimulated with intact, heat-inactivated *C. albicans* hyphae (left) or fragmented, heat-inactivated *C. albicans* hyphae (right). Fixed 1 hour post stimulation. Stained for MPO (green), *C. albicans* (red) and DAPI (blue). 0.8 µm z-stacks. Scale bars = 5 µm. (**c**) Release of NETs by human peripheral neutrophils stimulated with intact heat-inactivated WT *C. albicans* hyphae. Extracellular DNA stained with SYTOX 4 hours after stimulation (top). Brightfield images of neutrophils and heat-inactivated *C. albicans* (bottom). Scale bars = 50 µm. (**d**) Quantification of NET release by human peripheral neutrophils left unstimulated (Unst) or stimulated with heat-inactivated or untreated WT *C. albicans* hyphae, presented as $SYTOX^+$ events relative to total neutrophils. (**e**) Release of NETs by human peripheral neutrophils left unstimulated or stimulated with intact or fragmented WT *C. albicans* hyphae. Scale bars = 50 µm. (**f**) Quantification of NET release by human peripheral neutrophils left unstimulated or stimulated with intact or fragmented WT *C. albicans* hyphae. | Multiplicity of infection (MOI) = 10 (or fragments equivalent of MOI = 10 of intact hyphae). Statistics by one-way ANOVA followed by Tukey's multiple comparison post-test. *** $p \leq 0.001$ and **** $p \leq 0.001$. Data are representative of at least three independent experiments.

## *A. fumigatus hyphae and aggregates induce NETs*

Next, we investigated whether the size-dependent regulation of NET release was a general mechanism that applied to other microbes beyond *C. albicans. Aspergillus fumigatus* is a dimorphic fungus that can switch its morphology between small conidia and large hyphae. The conidia have a comparable size to *C. albicans* yeast particles (3-5 μm).

We tested NET induction by *Aspergillus fumigatus* in the presence and absence of plasma in the cell culture medium. *A. fumigatus* triggered NETosis in the presence but not in the absence of 3% plasma. Interestingly, in contrast to *C. albicans*, plasma did not induce *A. fumigatus* hyphal growth but triggered conidia to accumulate to large aggregates. These large aggregates stimulated the release of NETs (**Figure 4**). We pre-formed *A. fumigatus* hyphae in RPMI medium to confirm the importance of fungal size rather than the presence of plasma. Importantly, stimulation of neutrophils with the large filaments induced NET release in the absence of plasma (**Figure 4**).

These data confirm that size-dependent NET release applies to other fungal species and is not restricted to *C. albicans*. Furthermore, this process is not dependent on plasma or on the presence of fungal hyphae. It is instead dependent on the presence of microbial particles that are too large to be phagocytosed.

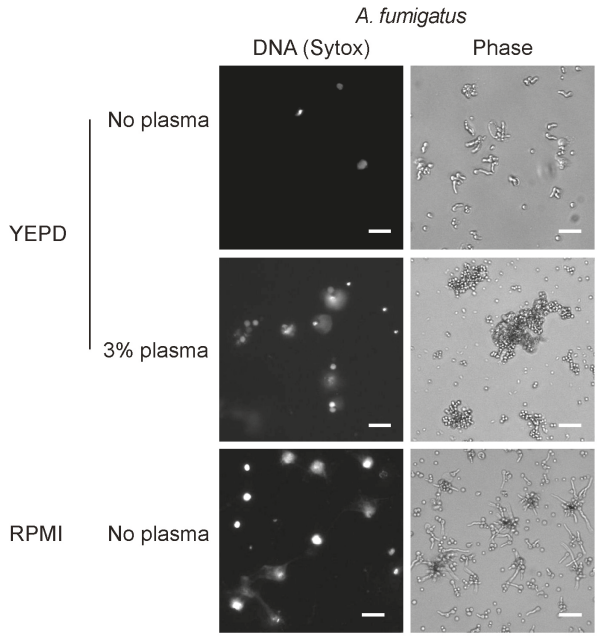


**Figure 4 | *A. fumigatus* filaments and aggregates induce NETs.** Release of NETs by human peripheral neutrophils stimulated with *A. fumigatus* in the absence (top) or presence (middle) of 3% plasma or *A. fumigatus* pre-formed hyphae (in RPMI) in the absence of plasma (bottom). *A. fumigatus* aggregates in the presence of plasma (middle). Extracellular DNA was stained with SYTOX 4 hours after stimulation (left). Brightfield microscopy of neutrophils and *A. fumigatus* (right). Scale bars = 50 µm. Data are representative of at least three independent experiments.

## *Single bacteria do not induce NETosis*

Next, we tested whether size-dependent NET release was restricted to fungi. Bacteria are small microbes that can easily be phagocytosed by neutrophils. According to the above findings the small size would make bacteria unsuitable NET-inducers.

We stimulated neutrophils with *Escherichia coli* and *Klebsiella pneumoniae*. In line with our previous findings, the small single bacteria did not elicit NET release (**Figure 5a** and **b**). Next we presented bacteria via the modified transwell described above, allowing for direct contact but preventing phagocytic uptake. Interestingly, bacteria that were presented in the

transwell induced NET release (**Figure 5a** and **b**), indicating that bacteria have the potential to activate NETosis when prevented from phagocytosis.

A more physiological approach to investigate this concept is the use of the Bacillus Calmette–Guérin (BCG) strain of *Mycobacterium bovis*. BCG forms irregular, large aggregates (Bernut, 2014; Ratliff, 1994). We investigated whether bacterial aggregation played a role in NET release. In our experiment a red fluorescent strain of BCG (BCG-dsRed) formed large as well as smaller aggregates. Interestingly, the size of the BCG aggregates strongly correlated with their capacity to induce NET release. Only large aggregates induced NETosis whereas smaller aggregates and single bacteria failed to trigger NET release. The smaller particles were taken up by phagocytosis (**Figure 5c** - **e**).

These data show that bacteria have the potential to induce NET release. However, their small size down-regulates this process. Preventing uptake of small bacteria by modified transwells or formation of large aggregates triggers release of NETs similar to large microbes.

Taken together, these *in vitro* experiments show for the first time that neutrophil antimicrobial responses are regulated. This regulation is neutrophil intrinsic. Neutrophils recognise the size of a microbe through whether or not it can be taken up. NETosis is an extracellular strategy that specifically targets large forms that cannot be eliminated intracellularly. Most fungi are di-morphic and germinate to larger hyphal forms for full pathogenicity. Bacteria are small and are phagocytosed rapidly. Our data show that bacteria do not trigger NET release, unless their uptake is prohibited. Formation of large aggregates in e.g. biofilms is a virulence mechanism of bacteria to avoid phagocytosis. Multiple bacterial virulence factors lead to abrogated phagocytosis, explaining literature describing NET release upon bacterial stimulation (Johnson and Criss, 2013; Juneau, 2015b).

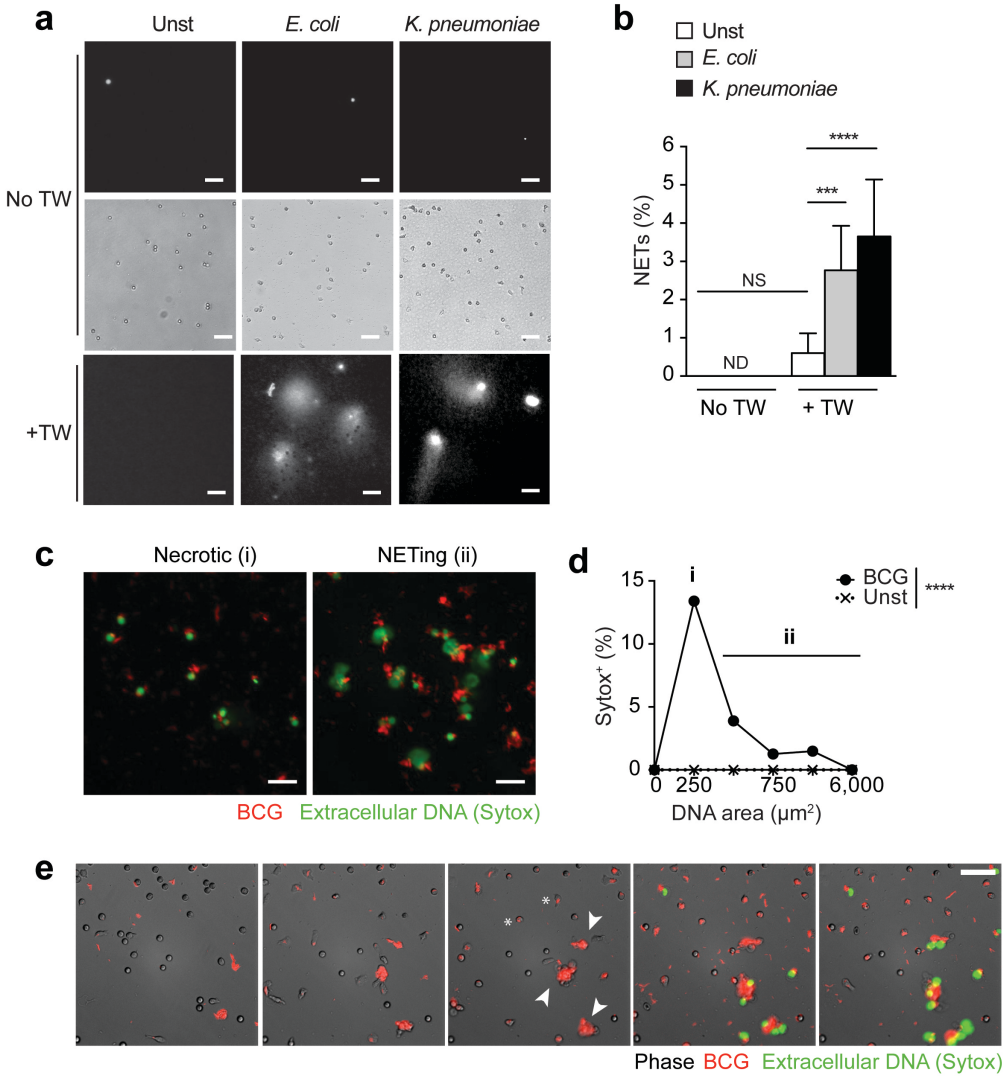


**Figure 5 | Single bacteria do not induce NETosis.** (**a**) Release of NETs by human peripheral neutrophils left unstimulated (Unst) or stimulated with *Escherichia coli* (strain DH5α) or *Klebsiella pneumoniae* (strain Kp52.145), directly (No TW, top and middle) or separated by a modified transwell to allow direct contact with the bacteria but prevent phagocytosis (+TW, bottom). Extracellular DNA was stained with SYTOX 4 hours after stimulation (top and bottom). Brightfield images of the neutrophils and bacteria (middle). Scale bars = 50 µm. (**b**) Quantification of NET release by human peripheral human peripheral neutrophils in response to BCG as described in (a), presented as $SYTOX^+$ events relative to total neutrophils. ND, not detectable. (**c**) Release of NETs by human peripheral neutrophils stimulated with BCG-DsRed (red), MOI=10. Extracellular DNA was stained with SYTOX (green) 4 hours after stimulation. Necrotic neutrophils responding to single BCG bacteria and small aggregates of BCG (i) and neutrophils undergoing NETosis in response to large aggregates of BCG (ii). Scale bars = 50 µm. (**d**) Quantification of necrotic neutrophils (i) and NET release (ii) by human peripheral neutrophils in response to BCG as described in (c) presented as $SYTOX^+$ events relative to total neutrophils. (**e**) Still images obtained from time-lapse video microscopy of human peripheral neutrophils stimulated with single bacteria (*) and large aggregates (arrowheads) of BCG-dsRed (red) in the presence of SYTOX (green). Scale bars = 150 µm. | Statistics by one-way ANOVA followed by Sidak's (b) or Tukey's (d) multiple comparison post-test. NS (not significant) $p > 0.5$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Data are representative of two independent experiments.

### 2.5.3 NETs have direct antifungal activity *in vitro*

Release of NETs was selectively triggered by large microbes such as *C. albicans* hyphae. However, the antimicrobial effect of NETs on hyphae was unknown. Therefore, we tested whether NETosis was required to control *C. albicans* hyphae *in vitro*.

First, we employed a growth inhibition assay that was based on the enzymatic activity of the live fungus. Live *C. albicans* metabolised the substrate 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) and produced a measurable yellow colour reaction (**Figure 6a**).

When *C. albicans* was incubated in the presence of neutrophils, viability was decreased compared to untreated *C. albicans*. At a multiplicity of infection (MOI) of 1, *C. albicans* growth was not completely controlled by 4 hours and the fungus continued to grow over night. At a decreased MOI of 0.1, *C. albicans* growth was completely inhibited and outgrowth was abrogated (**Figure 6b**), indicating that neutrophils are able to control *C. albicans* viability.

The enzymatic assay allowed us to investigate killing of *C. albicans* since it required a live fungus to metabolise the substrate. However, the disadvantage of this assay is that it only represents the effect of neutrophils on the entire *C. albicans* population. In order to dissect the effect of neutrophils on individual hyphal filaments, we employed time-lapse microscopy.

*C. albicans* WT hyphae showed a continued linear growth over 10 hours, reaching up to 30 times their original length under *in vitro* culture conditions. Addition of neutrophils to the cultures led to inhibited hyphal growth. This inhibition was directly dependent on the number of neutrophils present. Full growth inhibition was achieved at an MOI of 0.04 where on average 25 times more neutrophils than hyphal filaments were present (**Figure 6c**). To test the

importance of NET release in *C. albicans* killing, we dissolved NETs directly after release by the presence of DNase I in the culture medium. As before, *C. albicans* hyphae alone grew several times their original length, whereas the presence of neutrophils inhibited hyphal growth (**Figure 6d** and **e**). Importantly, hyphal growth inhibition was dependent on NETosis as it was partially recovered in the presence of DNase I (**Figure 6d** and **e**).

Since ROS-deficient mice succumb to infection with *C. albicans* due to their lack of NETosis, we tested whether ROS is required for direct hyphal growth inhibition. Therefore, we isolated bone marrow neutrophils from NADPH oxidase-deficient mice and investigated their anti-fungal properties.

Interestingly, *C. albicans* hyphae that were co-cultured with NADPH oxidase-deficient neutrophils showed hyphal growth comparable to untreated control hyphae. NADPH oxidase-deficient neutrophils did not exert any anti-fungal effect on *C. albicans* (**Figure 6f**).

Taken together, these data suggest that neutrophils directly kill *C. albicans* hyphae and that NETs are required for hyphal growth inhibition. Furthermore, the data underscore the importance for ROS during NET-mediated *C. albicans* fungal control.

**Figure 6 | NETs have direct antifungal activity *in vitro*.** (**a**) Graphic representation of XTT-based viability assay. *C. albicans* was left untreated (top) or co-incubated with human peripheral neutrophils (bottom), XTT substrate was added after indicated time-points. Live *C. albicans* metabolised substrate to yellow chromogenic substance, no reaction was seen in killed *C. albicans.* Absorbance measured at 450 nm. (**b**) Fraction of viable *C. albicans* WT hyphae after incubation with human peripheral neutrophils for indicated times, as compared to untreated *C. albicans* hyphae alone (dotted line). MOI = 1 (black bars) and 0.1 (white bars). (**c**) Brightfield microscopy of *C. albicans* WT hyphal outgrowth after 10 hours, left untreated (left panel) or incubated with increasing amounts of human peripheral neutrophils. Scale bar = 50 µm. (**d**) Brightfield microscopy of *C. albicans* WT hyphal outgrowth after 10 hours, left untreated (left panel) or incubated with human peripheral neutrophils, in the presence or absence of 50 U/ml DNase I. Scale bar = 50 µm.

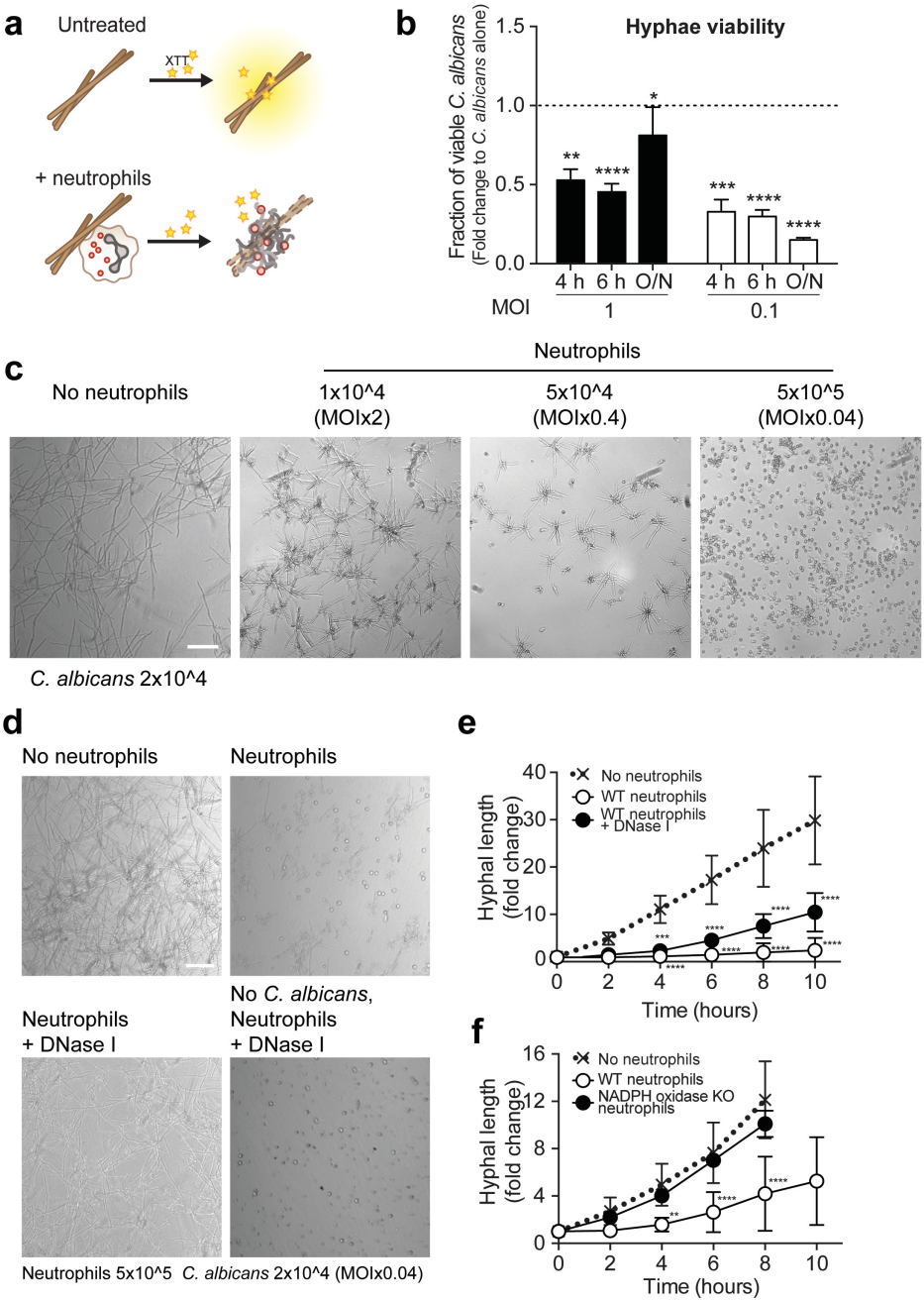


**Figure 6 (continued) |** (**e**) Quantitation of *C. albicans* WT hyphal outgrowth as in (d). Fold change as compared to hyphal length at 0 h. (**f**) Quantitation of *C. albicans* WT hyphal outgrowth over 10 hours left untreated or incubated with WT or NADPH oxidase KO mouse bone marrow neutrophils. | Statistics (a) by one-way ANOVA followed by Sidak's multiple comparison post-test. Error bars = SD, standard deviation. (d and e) compared to no neutrophils by two-way ANOVA followed by Dunnett's multiple comparison post-test. If nothing indicated = not significant (NS) $p > 0.05$. Otherwise: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Data are representative of at least three independent experiments.

### 2.5.4 NETs control hyphae *in vivo*

Our results showed that NETs were selectively released upon stimulation with large microbes and controlled *C. albicans* hyphae *in vitro*. Next, we tested whether NETs were released in a size-dependent manner *in vivo* and which role selective NETosis played during effective antifungal defence.

In a pulmonary infection model, C57BL/6 WT mice were inoculated intra-tracheally with *C. albicans* pre-formed WT hyphae and the *hgc1Δ* yeast locked strain. In line with the *in vitro* findings, we observed NET release in the lungs of mice infected with *C. albicans* WT hyphae. In contrast, animals infected with *C. albicans hgc1Δ* yeast particles did not release NETs in the lungs. NETosis was indicated by the presence of citrullinated histone 3 (citH3) (**Figure 7a**).

Similarly, infection with the lung pathogenic bacterium *Streptococcus pneumoniae* did not trigger NET release in the lung (**Figure 7b**) (Ellis, 2015), which confirmed the *in vitro* findings showing that bacteria are too small to trigger NETosis.

These findings suggest that NET release *in vivo* is dependent on microbe size.

After we established that NETosis was selectively induced by large extracellular microbes *in vitro* and *in vivo*, we tested whether size-dependent NETosis was crucial for sufficient antifungal defence *in vivo*.

Since MPO is required for the onset of NETosis and its absence leads to NET deficiency in mice and humans (Metzler, 2011), we tested the importance of NETosis for size-specific antimicrobial defence by pulmonary infection of MPO-deficient mice.

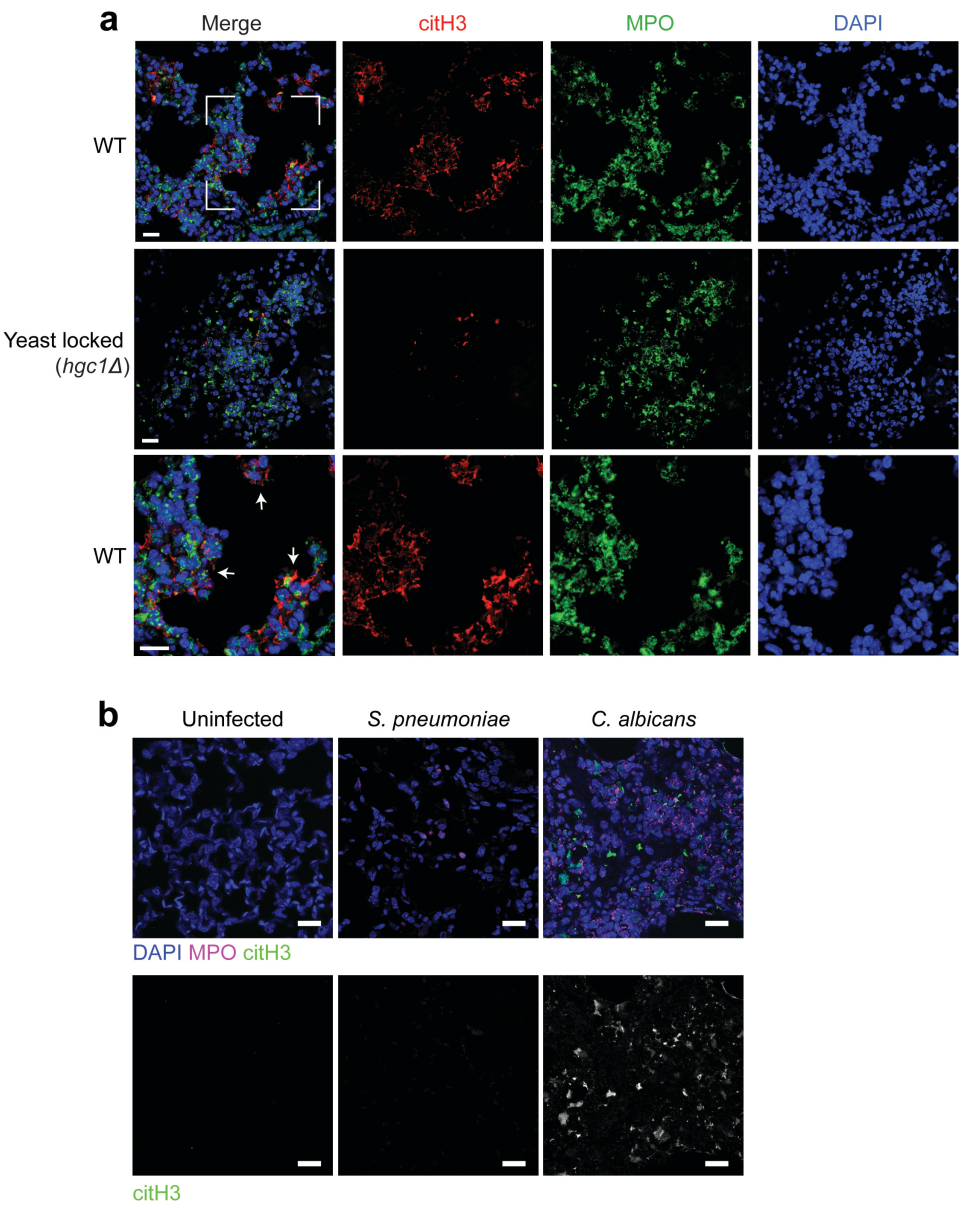


**Figure 7 | Hyphae but not yeast induce NETs *in vivo*.** (**a**) NET release (white arrows) in the lungs of wild-type (C57BL/6) mice 24 hours after intratracheal infection with 1 × $10^5$ colony-forming units (CFU) of *C. albicans* pre-formed WT hyphae or *hgc1Δ* yeast, assessed by immunofluorescence microscopy. Bottom row, enlargement of area outlined in top left image. (**b**) NET release in the lungs of wild-type (C57BL/6) mice after intranasal infection with 2x$10^7$ *Streptococcus pneumoniae* D39 or intratracheal infection with 1 × $10^5$ colony-forming units (CFU) of *C. albicans* pre-formed WT hyphae, assessed by immunofluorescence microscopy. | Stained for citrullinated histone H3 (citH3; red), MPO (green) and DNA (DAPI, blue). Scale bars, 20 µm. Images are representative of two independent experiments. (b) Infection courtesy of Dr Gregory T Ellis.

We infected C57BL/6 WT and MPO-deficient (ΔMPO) mice intra-tracheally with WT *C. albicans* and the *hgc1Δ* yeast-locked strain. We monitored the weight and general health of the mice over the course of 6 days. Initially, all mice lost weight due to the stress of the infection. WT mice recovered quickly and regained weight 48 hours after infection with either strains of *C. albicans* (**Figure 8a**). ΔMPO mice recovered quickly from the infection with the yeast-locked *hgc1Δ* strain of *C. albicans*. In contrast, NET-deficient ΔMPO mice succumbed rapidly to infection with the hyphal-containing WT strain of *C. albicans*. These mice lost up to 30% of body weight 6 days post infection (**Figure 8a**). Furthermore, WT mice cleared infection completely by day 6 post infection, as indicated by the absence of *C. albicans* colony forming units (CFU) in the lung and spleen of these mice. In contrast, high numbers of *C. albicans* CFU were present in the lungs of the NET-deficient ΔMPO mice. Furthermore, the infection in these mice spread systemically, as indicated by the presence of *C. albicans* CFU in the spleen on day 6 post infection (**Figure 8b**).

To verify that the selective susceptibility of mice towards large WT hyphae was not caused by an attenuation of the *Δhgc1* yeast-locked *C. albicans* strain, we employed NADPH oxidase-deficient mice. Since ROS production by NADPH oxidase is crucial for NETosis and killing by phagocytosis, these mice are highly susceptible to infection (Ermert, 2009).

We infected C57BL/6 WT and NADPH oxidase-deficient mice with WT and *hgc1Δ* yeast-locked *C. albicans*. As seen previously, WT mice cleared infection with both fungal strains, indicated by rapid weight regain. Interestingly, NADPH oxidase-deficient mice succumbed to the infection with both WT and *hgc1Δ* yeast-locked *C. albicans* (**Figure 8c**), indicating that the *Δhgc1* yeast-locked strain is not attenuated.

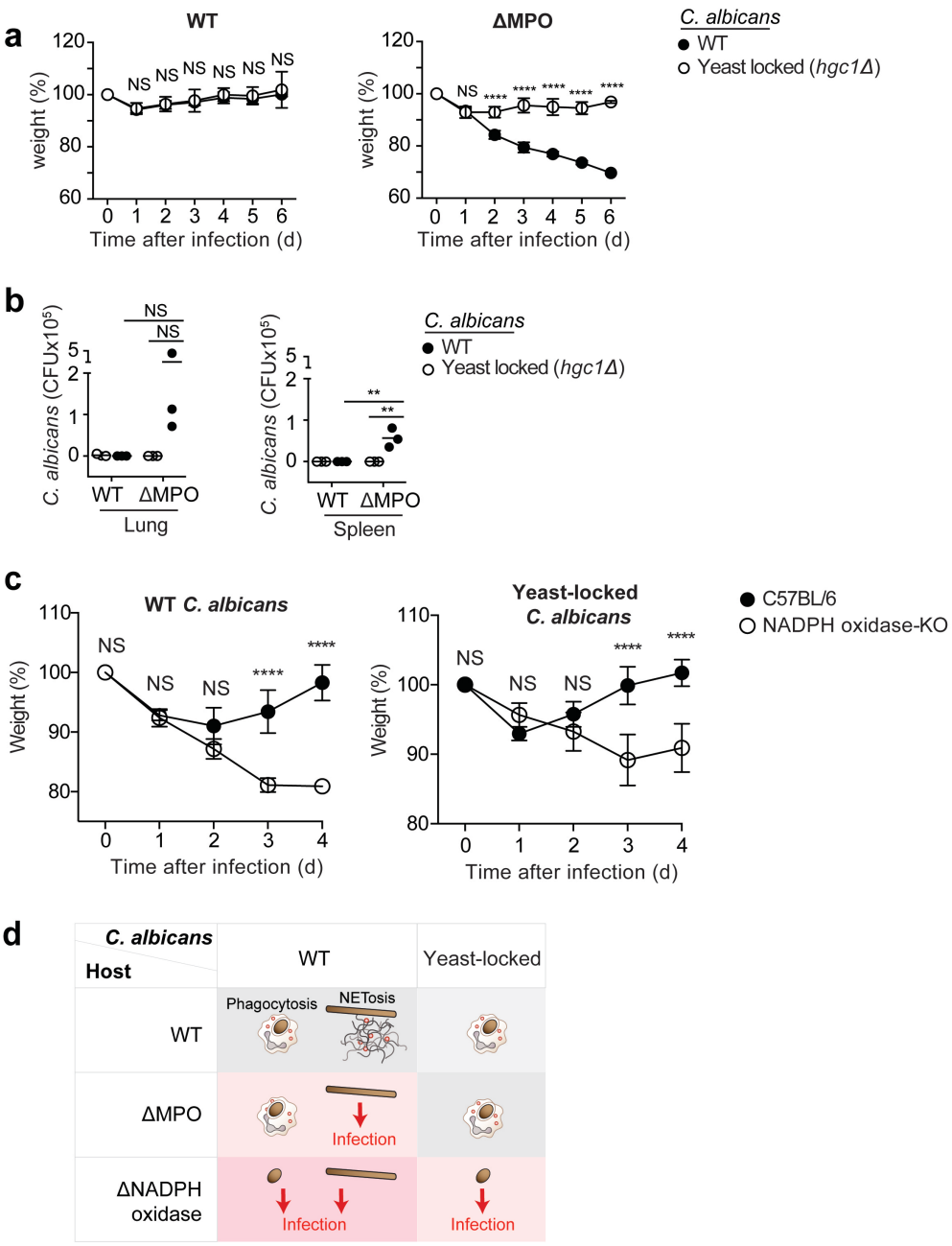


**Figure 8 | ROS-deficient mice succumb to infection with WT *C. albicans*.** (**a**) Weight of C57BL/6 wild-type mice (WT; n = 6) and MPO-deficient mice (ΔMPO; n = 5) infected with 1 × $10^4$ colony-forming units (CFU) of wild-type or *hgc1Δ C. albicans*, presented relative to starting weight (day 0), set as 100%. (**b**) *C. albicans* load in the lungs and spleen of mice 6 days after infection as in (a). Each symbol represents an individual mouse (n = 3 mice per group). (**c**) Weight of C57BL/6 WT and NADPH oxidase KO mice after infection with 1x$10^4$ colony-forming units (CFU) of WT and *hgc1Δ* yeast-locked *C. albicans* (n=5). Weight normalized to starting weight at day 0. (**d**) Overview of antimicrobial strategies of WT, ΔMPO and ΔNADPH oxidase mice after stimulation with WT or *Δhgc1* yeast-locked *C. albicans*. | Statistics (a and c) by two-way ANOVA, followed by Sidak's multiple comparison post test; (b) by two-way ANOVA, followed by Tukey's multiple-comparison post-test. Small horizontal lines indicate the mean. NS = not significant p > 0.05, ** p ≤ 0.01. **** p ≤ 0.0001. Data are representative of two independent experiments. Error bars = SD, standard deviation.

Taken together, these findings indicate that NET release is an important antimicrobial strategy, specifically targeted at large pathogens. Lack of this extracellular defence mechanism renders mice susceptible to prolonged infection with microbes that cannot be cleared intracellularly (**Figure 8d**). MPO deficiency is a good way to model NET deficiency as indicated by the phenotype of MPO-deficient patients (Metzler, 2011). However, since MPO is also expressed in subsets of macrophages (Klebanoff, 2005), it cannot be completely excluded that MPO deficiency also impairs other NET-unrelated functions *in vivo*. NADPH-deficient mice model chronic granulomatous disease (CGD). NADPH oxidase deficiency and subsequent lack of ROS production inhibits NETosis as well as killing by phagocytosis. Both strategies are required for an effective immune response, each targeting microbes of different size.

### 2.5.5 Dectin-1 is a negative regulator of NETosis

*Blocking phagocytic receptors upregulates NET release in vitro*

Since phagocytosis and NETosis seemed to be two complementing strategies in antimicrobial defence, we further investigated their interplay. Dectin-1 is the main fungal-specific phagocytic receptor. It recognises β-glucan in the fungal cell wall and triggers uptake of small fungal particles. Therefore, we tested the influence of dectin-1-mediated phagocytosis on NET release.

Inhibiting dectin-1 in neutrophils with a blocking antibody *in vitro* led to a great reduction of phagocytosis as compared to unblocked control cells. This was indicated by a reduced uptake rate of yeast particles as well as a reduced total number of phagocytosed particles (**Figure 9a**). Interestingly, at the same rate as phagocytosis decreased, the release of NETs increased. Neutrophils with blocked dectin-1 released NETs in response to stimulation with *hgc1Δ C. albicans* yeast particles, which were otherwise unable to induce NETosis in the absence of dectin-1 blocking. Stimulation of dectin-1-blocked neutrophils with WT *C. albicans* hyphae led to even further increased

NETosis as compared to untreated dectin-1-competent neutrophils (**Figure 9b**). This is potentially due to the blocked uptake of smaller hyphal fragments present in the fungal preparation. Interestingly, over time fully phagocytosing neutrophils died of necrosis with condensed nuclei. In contrast, dectin-1-blocked neutrophils showed reduced signs of phagocytosis. Their nuclei decondensed and NETs were formed (**Figure 9c**), indicating a repressive effect of dectin-1 on NETosis.

Next we investigated whether NETosis was specifically regulated by dectin-1 or whether phagocytosis was a general repressor of NET release. Therefore, we tested the capacity of phagocytic receptors to modulate NET release. Blocking the Fcγ receptor (FcγR) increased the amount of NETs released upon stimulation with WT *C. albicans* hyphae by two fold. These results were later confirmed by another group (Aleman, 2016). Similarly, blocking the mannose receptor (MR) or the complement-dependent phagocytic receptor CR3 increased NET release upon stimulation with *C. albicans* hyphae as compared to unblocked neutrophils (**Figure 9d**).

These data indicate that phagocytosis inhibits NET release independent of the initiating receptor. Therefore, naturally occurring phagocytosis is a master regulator of NETosis. This regulation mechanism attributes to the selectivity of the two antimicrobial strategies phagocytosis and NETosis for small and large microbes, respectively. These results also indicate that recognition of *C. albicans* by dectin-1, FcγR, MR or CR3 is not required for the initiation of the NETosis pathway. Blocking these receptors did not abrogate, but increase NET release upon stimulation with *C. albicans*.

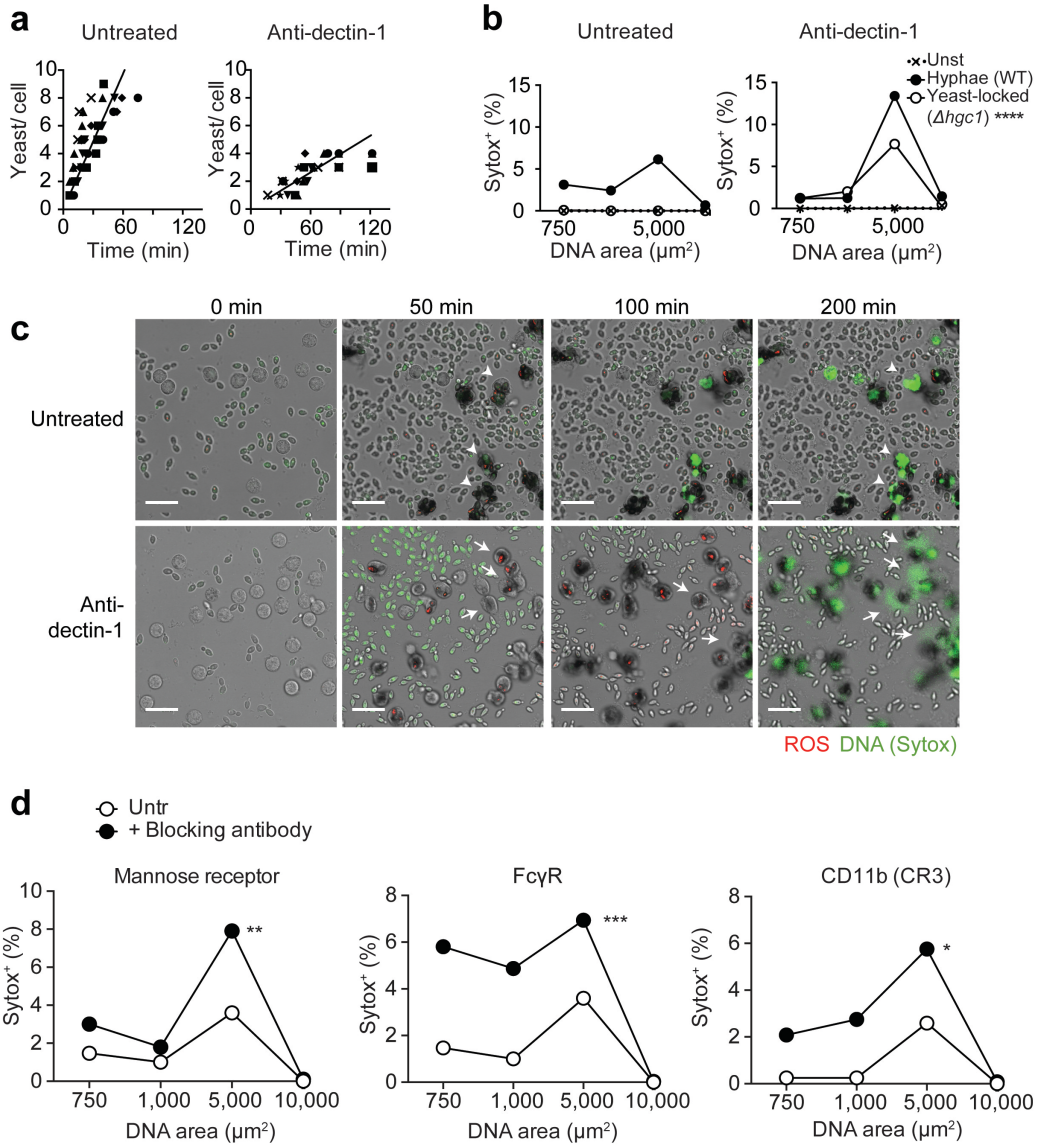


**Figure 9 | Blocking phagocytic receptors upregulates NET release *in vitro*.** (**a**) Phagocytosis of *hgc1Δ C. albicans* yeast (MOI = 40) by neutrophils (n = 10 per condition) left untreated (left) or treated with 10 μg/ml anti-dectin-1 blocking antibody (right); phagocytosed yeast particles per cell were quantified over 2 hour by live microscopy (diagonal line: trend line fitted to data). (**b**) NET release by peripheral human neutrophils left untreated (left) or treated with 10 μg/ml anti-dectin-1 blocking antibody (right), left unstimulated or stimulated with *C. albicans* hyphae (WT) or *hgc1Δ* yeast (*hgc1Δ*) (MOI = 10), assessed 4 hours after stimulation, presented as $SYTOX^+$ events relative to total neutrophils. (**c**) Time-lapse microscopy of live human peripheral neutrophils left untreated (top) or treated with 10 μg/ml anti-dectin-1 blocking antibody (bottom) and stimulated with heat-inactivated *hgc1Δ C. albicans* yeast (MOI = 40); arrowheads indicate incomplete decondensation; arrows indicate NET release. Confocal images were obtained every 30 s. Scale bars = 20 μm (**d**) NET release by peripheral human neutrophils left untreated (Untr) or treated with blocking antibodies against Mannose receptor (10 μg/ml, left), FcγR (0.5 μg/ml, middle) or CR3 (10 μg/ml, right), stimulated with WT *C. albicans* hyphae (MOI = 10), assessed 4 hours after stimulation, presented as $SYTOX^+$ events relative to total neutrophils. | (b and d) Statistics by one-way ANOVA followed by Sidak's multiple-comparison post-test * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Data are representative of at least three independent experiments.

## *Dectin-1 KO mice have increased NET release in vivo*

Blocking phagocytosis *in vitro* led to NET release upon stimulation with small yeast and to increased NETosis upon stimulation with large hyphae. Therefore, we tested whether phagocytosis was also relevant for regulating NETosis *in vivo*, using dectin-1-deficient mice.

We infected WT and dectin-1-deficient mice with WT *C. albicans* intratracheally and observed NET release in the lung as indicated by the presence of citrullinated histone 3 (citH3). Interestingly, dectin-1 deficient mice showed a marked increase in NET release upon infection with *C. albicans* as compared to WT mice (**Figure 10a**). Furthermore, infection of dectin-1-deficient mice with the fungus *A. fumigatus* led to increased NET release in the lungs as compared to WT mice (**Figure 10b**).

These data further corroborate the selective release of NETs in response to particles that cannot be phagocytosed. Mice lacking the phagocytic receptor dectin-1 show a greatly increased NET response upon encounter with *C. albicans*. Similarly, *A. fumigatus* triggered increased NET release in dectin-1-deficient mice, indicating that regulation of NETosis by phagocytosis is a general mechanism and not restricted to *C. albicans*.

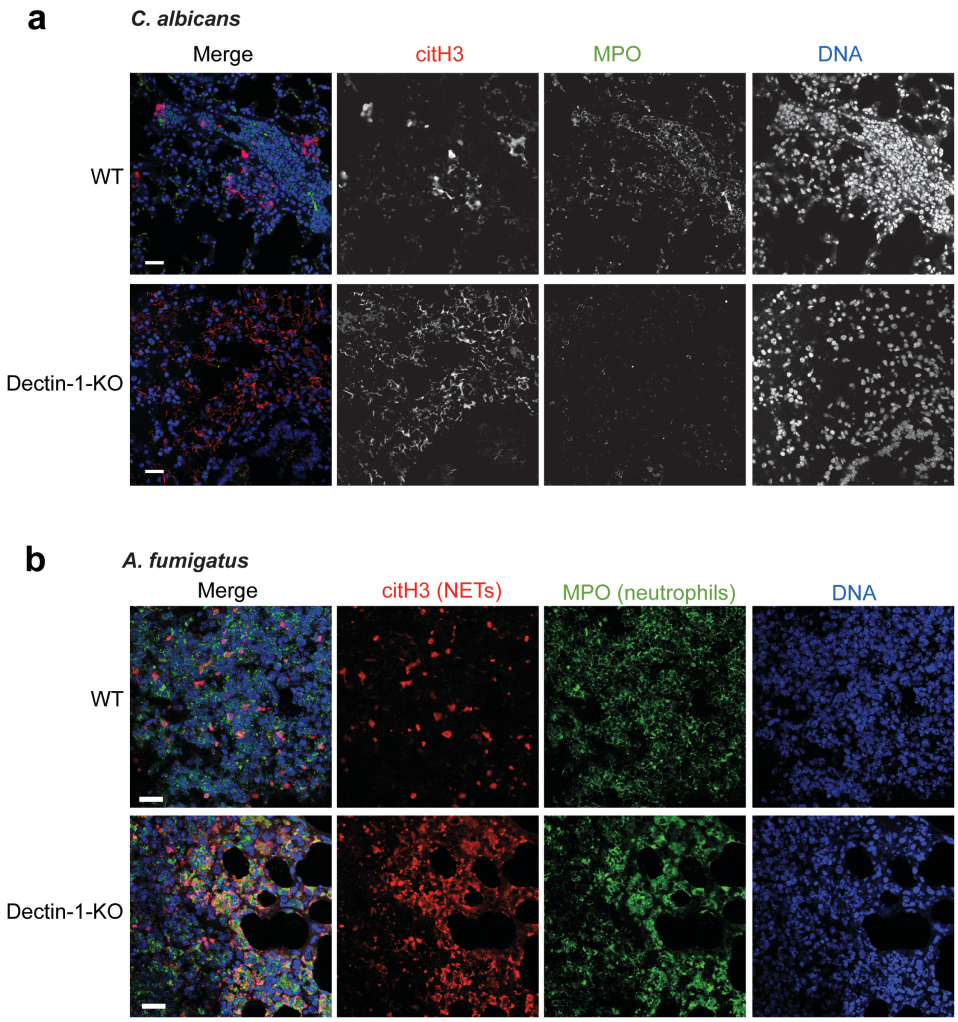


**Figure 10 | Dectin-1 KO mice have increased NET release *in vivo*.** (**a**) NET release in lungs of WT (C57BL/6) and dectin-1 deficient mice infected with $1x10^5$ CFU WT *C. albicans* 24 hours post infection. (**b**) NET release in the lungs of C57BL/6 WT mice and dectin-1-deficient mice (Dectin-1-KO) infected intratracheally with $1 \times 10^6$ colony forming units (CFU) of *A. fumigatus*, assessed 48 hours later. | Immunofluorescence staining for DNA (DAPI, blue), MPO (green) and citrullinated histone H3 (citH3, red), analysed by immunofluorescence confocal microscopy. Scale bars = 20 µm. Images are representative of two independent experiments. | (b) Staining courtesy of Dr Qian Wang.

### 2.5.6 Selectivity of NETosis is not regulated through signalling or ROS

Our data indicated that dectin-1 acted as negative regulator of NETosis, driving size-dependent NET release. However, it was unclear how this regulation was achieved mechanistically. Therefore, we investigated whether dectin-1-mediated signalling was activated differentially by large and small particles.

We tested the activation of Syk kinase, which is phosphorylated downstream of dectin-1 (Drummond, 2011). Since fungi signal through different TLRs, we investigated whether *C. albicans* yeast and hyphae initiated differential TLR signalling. Therefore, we tested the phosphorylation of the TLR-activated ERK kinase pathway (Bourgeois and Kuchler, 2012; Romani, 2011).

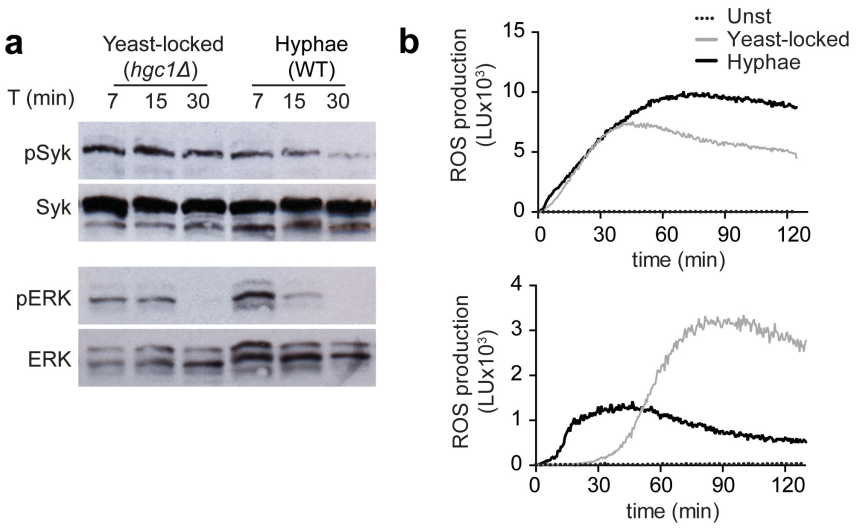


**Figure 11 | Selectivity of NETosis is not regulated through signalling or ROS.** (**a**) Syk and ERK kinase activation in human peripheral neutrophils stimulated with *C. albicans* WT hyphae or *hgc1Δ* yeast-locked mutant for the indicated times and assessed by immunoblotting. Syk/pSyk = 72 KDa, ERK/pERK = 42 + 44 kDa bands. (**b**) Production of reactive oxygen species (ROS) by human peripheral neutrophils after stimulation with *C. albicans* WT hyphae or *hgc1Δ* yeast-locked mutant. Unst, unstimulated; LU, luminescence units. Data are representative of at least three independent experiments.

We stimulated human peripheral neutrophils with *C. albicans* WT hyphae and *hgc1Δ* yeast and probed for phosphorylation of Syk and ERK kinase by immunoblotting. Interestingly, the expression or phosphorylation of Syk or ERK did not differ between neutrophils stimulated with either *C. albicans* strain (**Figure 11a**). These results indicate that both growth forms equally activate CLR and TLR signalling in neutrophils.

Since ROS is crucial for NETosis, we investigated the production of ROS upon stimulation with WT *C. albicans* hyphae and *hgc1Δ* yeast. We observed strong donor variability with varying ROS profiles upon stimulation with hyphae or yeast respectively (**Figure 11b**) (Two representative examples are shown). The amount of ROS produced after stimulation with either growth form did not correlate with the release of NETs.

These results indicate that neither activation of known antifungal signalling pathways such as Syk or ERK signalling, nor ROS levels correlate with the selective release of NETs. Therefore, the regulating mechanism of dectin-1 is not driven by its function as signalling receptor.

### 2.5.7 Phagocytosis inhibits NETosis by sequestering NE

#### *Phagosome maturation regulates NETosis*

Phagocytic receptors negatively regulated NETosis and inhibition of phagocytosis increased NET release. This regulation was independent of receptor signalling, indicating that downstream processes during phagocytosis might be involved. Phagosome maturation in neutrophils is dependent on the fusion of the phagosome with neutrophil granules, a step that requires actin polymerisation. This fusion allows the delivery of antimicrobial granule content to the phagosome for intracellular killing (Mollinedo, 2006; Nordenfelt and Tapper, 2011). We tested whether inhibition of phagosome maturation was important for the regulation of NETosis.

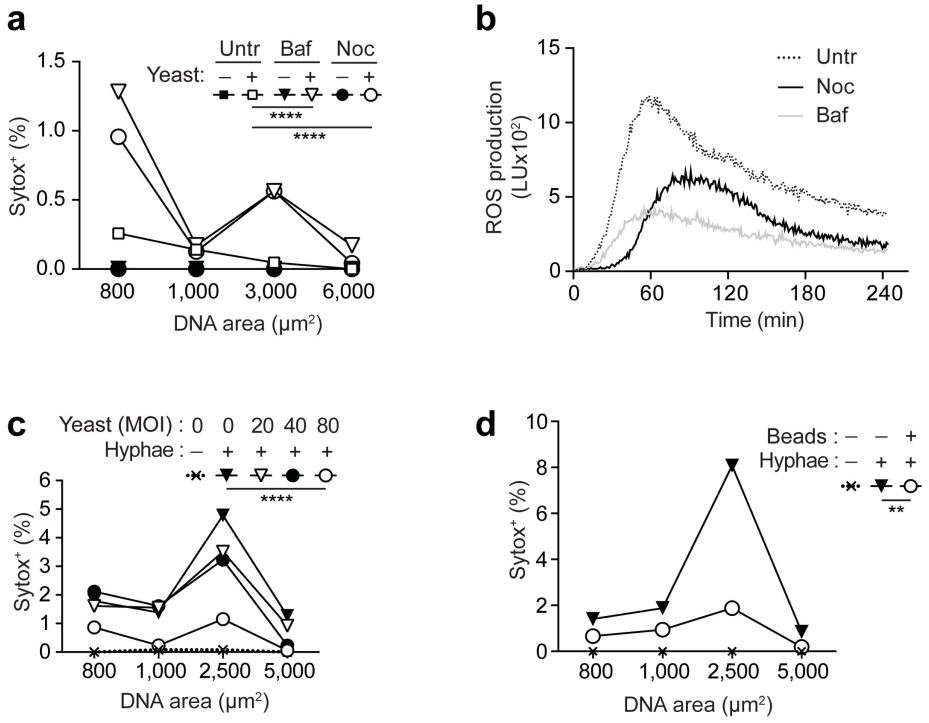


**Figure 12 | Blocking phagosome maturation increases NETosis.** (**a**) NET release by human peripheral neutrophils left untreated (Untr) or treated with 1 µM Bafilomycin A1 (Baf) or 2.5 µM Nocodazole (Noc), left unstimulated (−) or stimulated with *hgc1Δ C. albicans* yeast (+), MOI = 10, presented as SYTOX$^+$ events relative to total neutrophils. (**b**) Production of reactive oxygen species (ROS) by human peripheral neutrophils left untreated or treated with Bafilomycin A1 or Nocodazole and stimulated with *hgc1Δ C. albicans* yeast, MOI = 10. LU = luminescence units. (**c**) NET release by neutrophils pre-incubated for 1 h with *hgc1Δ C. albicans* yeast at an MOI of 0, 20, 40 or 80 and then left unstimulated (−) or stimulated with *C. albicans* hyphae (+), MOI =10, presented as SYTOX$^+$ events relative to total neutrophils. (**d**) NET release by neutrophils pre-incubated for 1 hour with 0.1 µm polystyrene beads and then stimulated with *C. albicans* hyphae, MOI =10. | Statistics by two-way ANOVA, followed by Sidak's multiple comparison posttest: ** $p \leq 0.01$, **** $p \leq 0.0001$. Data are representative of at least three independent experiments.

Therefore, we pre-treated neutrophils with Nocodazole, an inhibitor of microtubule formation, or with Bafilomycin, an inhibitor of phagosome acidification. Surprisingly, both treatments led to induction of NET release upon stimulation with *C. albicans* yeast particles, which did not trigger NETosis in the absence of those inhibitors (**Figure 12a**). Importantly, treatments with neither Bafilomycin nor Nocodazole led to markedly

increased ROS production. Instead, ROS were slightly reduced (**Figure 12b**). Therefore, NETosis was not induced by increased ROS production due to prolonged microbe presence in the phagosome.

These data indicated that phagosome maturation through fusion of neutrophil granules regulated NETosis. Since inhibition of phagosome maturation increased NETosis, we tested whether forcing phagocytosis would abrogate NET release.

Therefore, we pre-treated neutrophils with increasing amounts of *C. albicans* yeast particles or polystyrene beads and subsequently re-stimulated the neutrophils with *C. albicans* WT hyphae. The small particles were readily phagocytosed by the neutrophils (data not shown). Interestingly, neutrophils that had phagocytosed beads or yeast particles showed reduced capacity to respond with NETosis upon stimulation with *C. albicans* hyphae. The reduction of NET release correlated directly with the amount of yeast particles that were phagocytosed before (**Figure 12c** and **d**).

These results indicate that NET release is dose-dependently linked to the number of particles that are phagocytosed and the phagosomes that are formed in the process. A high number of phagocytosed particles completely abrogate NET release in response to an otherwise potent NET stimulus, whereas blocking of phagosome maturation releases the negative regulation and increases NETosis. We present a novel mechanism in which phagocytosis is a direct negative regulator of NETosis.

### *Phagocytosis sequesters NE and prevents translocation to the nucleus*

Fusion of preferentially azurophilic granules during phagosome maturation delivers granule content, such as NE, into the phagosome. During NET release, NE is required to translocate into the nucleus where it cleaves histones and initiates the decondensation of chromatin. Therefore, we

investigated whether NE localisation in stimulated neutrophils regulated size-specific NETosis.

Upon stimulation of human neutrophils with WT *C. albicans* hyphae up to 80% of the total NE translocated into the nucleus by about 4 hours post stimulation (**Figure 13a** and **b**). This finding was in line with previous reports (Papayannopoulos, 2010). In contrast, neutrophils that were stimulated with *hgc1Δ* yeast-locked *C. albicans* phagocytosed yeast particles and their nucleus was almost completely devoid of NE (**Figure 13a** and **b**).

In order to establish where NE was localised after phagocytosis, we stimulated human neutrophils with *C. albicans hgc1Δ* yeast particles and subsequently stained for the markers of phagosome maturation CD63 and p40. Interestingly, in phagocytosing neutrophils NE was associated with the phagosome rather than the nucleus (**Figure 13c**). Furthermore, staining of the *C. albicans* yeast particles co-localised directly with phagosomal stainings CD63 and p47, confirming that the yeast particles were located inside the phagosome (**Figure 13d** and **e**).

To test the role of dectin-1 in NE translocation, we stimulated neutrophils with small yeast particles. Importantly, these neutrophils did not form fully functional phagosomes and NE translocation to the nucleus was restored (**Figure 13f**).

In summary, in phagocytosing neutrophils NE does not translocate into the nucleus, but is found in the phagosome. Blocking phagocytosis restores NE translocation. Therefore, phagocytosis-driven competition for NE is the main regulator of selective NET release.

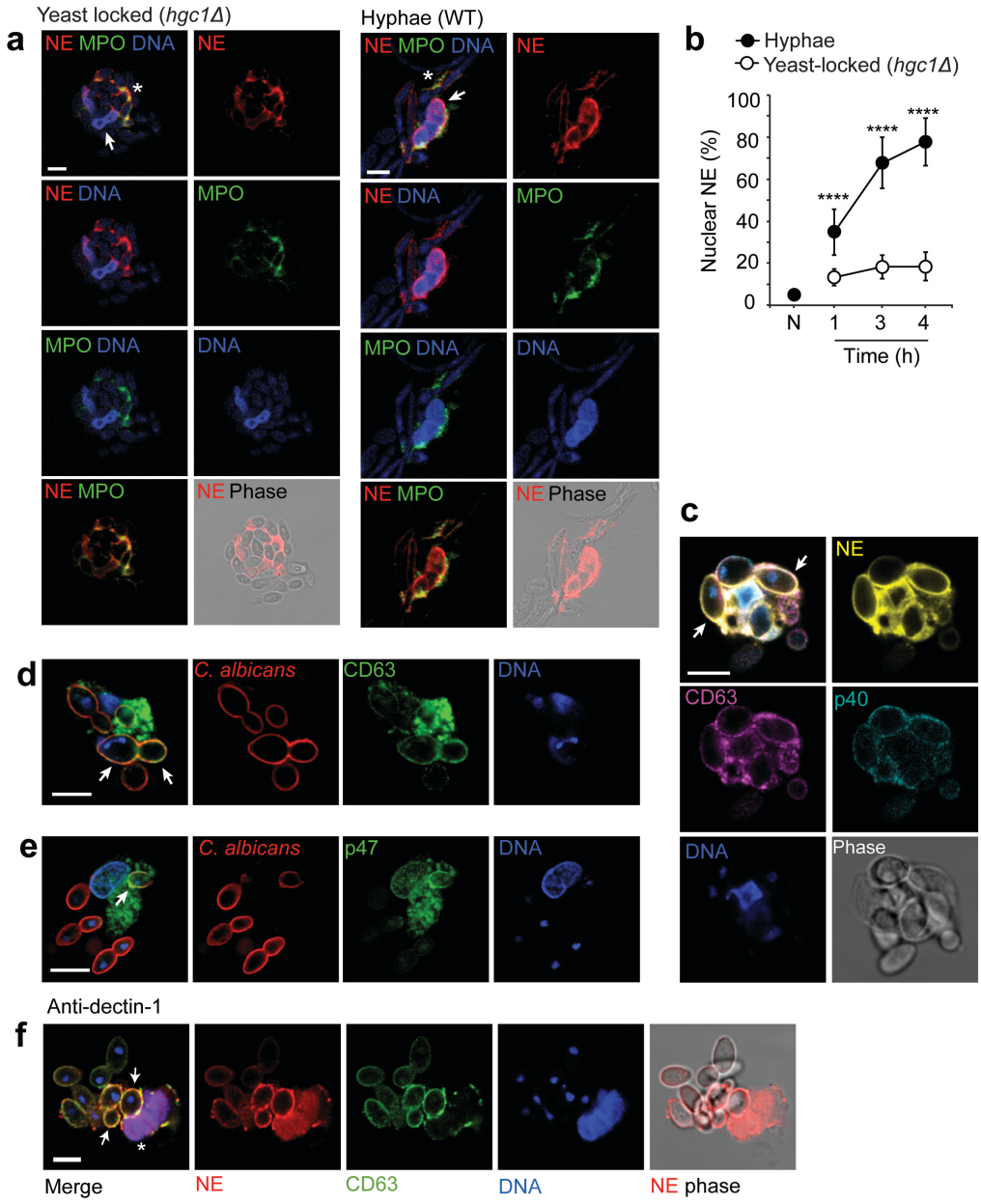


**Figure 13 | Phagocytosis sequesters NE and prevents translocation to the nucleus.** (**a**) Localization of NE (red) and MPO (green) in human peripheral neutrophils 1 hour after stimulation with *C. albicans hgc1Δ* yeast (left) or WT hyphae (right), assessed by immunofluorescence via confocal microscopy of an optical section spanning the centre of the cell. Arrows indicate the nucleus. * = colocalisation of NE with MPO. Bottom right (Phase), phase-contrast microscopy. (**b**) Localization of NE to the nucleus in naive neutrophils (N) and in neutrophils treated as in (a), presented as nuclear NE relative to total NE per cell (multiple confocal sections of 15–20 cells per condition). Statistics by two-way ANOVA, followed by Sidak's multiple comparison posttest: **** $p \leq 0.0001$. Error bars = SD, standard deviation. Data are representative of three independent experiments. (**c**) Localization of NE (yellow), CD63 (magenta), p40 (cyan) to the phagosome (arrows) and DAPI-stained DNA (blue) in human peripheral neutrophils 1 hour after stimulation with *hgc1Δ C. albicans* yeast. | Images are representative of three independent experiments.

### 2.5.8 Deregulated NET release promotes pathology

*Dectin-1 KO mice succumb to NET-mediated tissue damage*

NETosis is important for antimicrobial defence but has also been implicated in host tissue pathology (Narasaraju, 2011; Papayannopoulos, 2011). Since, dectin-1 deficiency and reduction in phagocytosis led to increased NET release, we investigated whether this de-regulated NETosis influenced antifungal defence or immune pathology. We employed a pulmonary infection model with a high dose of *C. albicans* yeast particles, where intracellular defence mechanisms would be sufficient to combat this infection in WT mice.

We infected WT and dectin-1-deficient mice with a high dose of *C. albicans hgc1Δ* yeast-locked particles and observed the animals over a course of 5 days. In parallel, we treated the mice with an NE inhibitor (NEi) to prevent NET formation or the tissue repair factor amphiregulin (AREG) (**Figure 14a**). Untreated WT and dectin-1-deficient mice succumbed rapidly to the high infectious load and died by day 2 post infection. Surprisingly, dectin-1-deficient mice treated with NEi were nearly completely rescued. In contrast, WT mice remained mainly unaffected by NEi treatment and succumbed by day 3 post infection. Treatment of the dectin-1-deficient mice with the tissue repair factor AREG led to alleviation of the phenotype and rescued around 50% of the infected animals, indicating that NETosis mediated pathology in these animals. WT mice remained mainly unaffected by AREG treatment (**Figure 14b**). Furthermore, WT mice produced large amounts of TNFα in the

**Figure 13 (continued) |** (**d** and **e**) Localization of CD63 (green) (d) or p47 (green) (e) and DAPI-stained DNA (blue) in human peripheral neutrophils 1 hour after stimulation with *hgc1Δ C. albicans* yeast (red). Scale bars (a,c,d and e) = 5 µm. (**f**) Localization of NE to the phagosome (arrows) or nucleus (*) in human peripheral neutrophils stimulated for 1 hour with *hgc1Δ C. albicans* yeast in the presence of anti-dectin-1 blocking antibody. Stained for NE (red), CD63 (green) and DNA (DAPI; blue). Scale bars = 5 µm. | (a and c - f) Stainings courtesy of Ms Aleksandra Lubojemska.

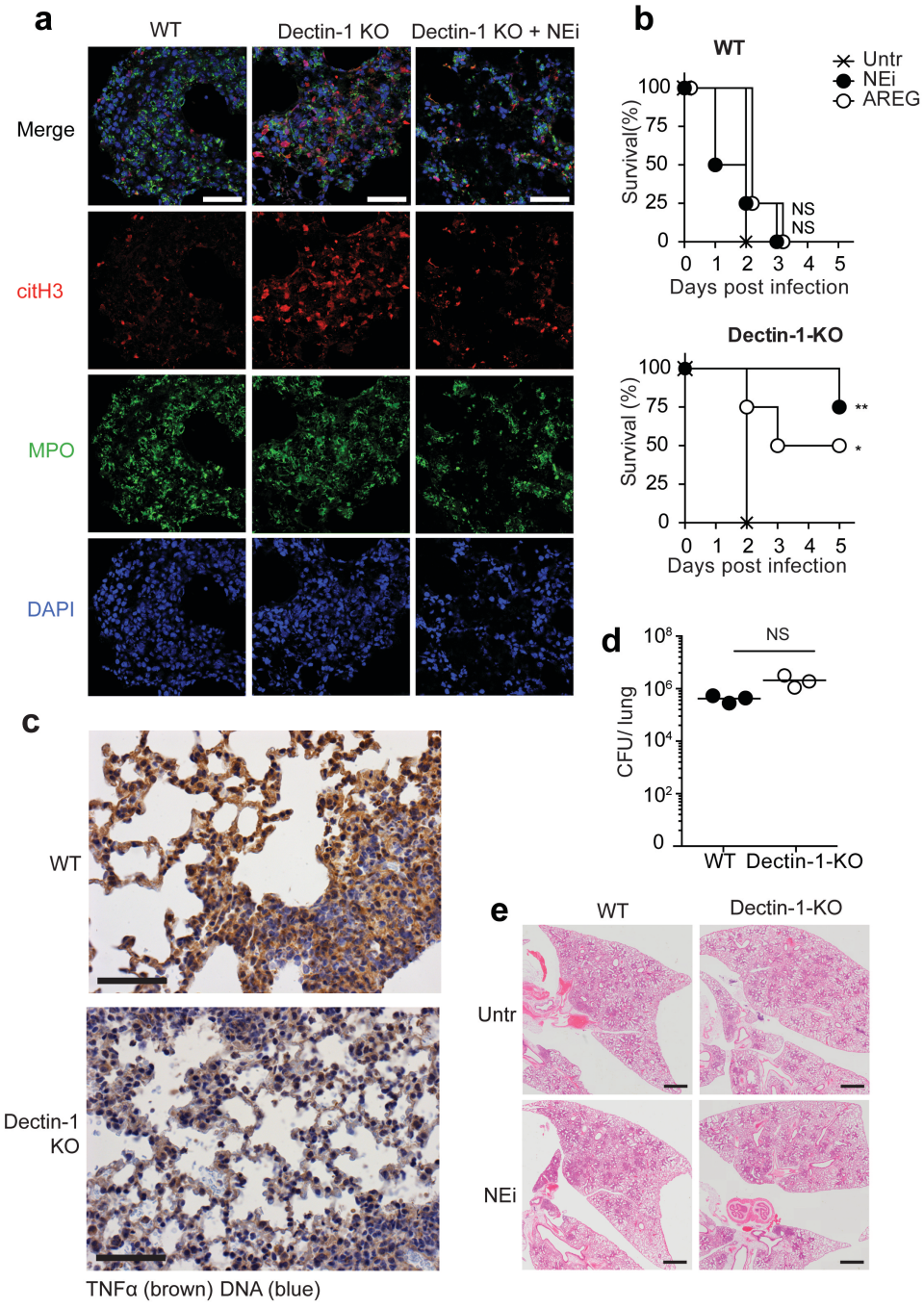


**Figure 14 | Dectin-1 KO mice succumb to NET-mediated pathology.** (**a**) NET release in lungs of wild-type and dectin-1-deficient mice left untreated (Untr) (left and middle) or treated with 2.5 µg/ g mouse of the NE inhibitor (+ NEi, right), infected intratracheally with $3 \times 10^6$ colony forming units (CFU) *hgc1Δ C. albicans* yeast, assessed 24 h later by immunofluorescence confocal microscopy of lung sections stained for DNA (DAPI; blue), citrullinated histone H3 (citH3, red) and MPO (green). Scale bars = 50 µm. (**b**) Survival of C57BL/6 WT mice and dectin-1-deficient mice (n = 4 per group) left untreated or treated with 2.5 µg/ g mouse of the NE inhibitor (NEi) or 0.4 µg/ g mouse of amphiregulin (AREG), infected intratracheally with $1 \times 10^7$ CFU of *hgc1Δ C. albicans* yeast. Statistics by log-rank (Mantel-Cox) test, followed by Tukey's multiple comparison post test: NS = not significant $p > 0.05$, * $p \leq 0.01$, ** $p \leq 0.001$.

lungs. This pro-inflammatory cytokine storm was most likely caused by septic shock in these mice due to the high dose of infectious material. Dectin-1 KO mice did not show any signs of TNFα production in the lungs (**Figure 14c**). These results are in agreement with the previous findings that dectin-1-deficient mice are unable to mount a sufficient pro-inflammatory response (Taylor, 2007). Therefore, dectin-1-deficient mice did not succumb to septic shock. Furthermore, fungal load in the lung and recruitment of neutrophils to the infected lungs was comparable in WT and dectin-1 mice (**Figure 14d** and **e**).

To further investigate whether increased NETosis caused pathology in dectin-1-deficient mice, we analysed lung histology after the infection. Interestingly, dectin-1-deficient mice showed increased tissue damage as compared to WT animals. This was indicated by haemorrhagic bleeding and fibrin deposition in the lung. Importantly, these pathologic symptoms could be alleviated by inhibiting NETosis with NEi treatment (**Figure 15a** and **b**).

In summary, these data indicate that NET-regulation in situations where microbes can be eliminated intracellularly limits NET-mediated immune pathology. In mice lacking dectin-1, reduced phagocytosis leads to increased NET release. The disruption of the mechanism, regulating size-dependent NETosis inflicts damage to the surrounding tissues and can potentially lead to the death of the affected host (**Figure 15c**).

**Figure 14 (continued) |** (**c**) TNFα levels in lung sections of C57BL/6 WT and dectin-1 deficient mice infected with 3 x $10^6$ CFU of *hgc1Δ C. albicans* yeast 36 hours post infection, stained by immunohistochemistry with anti-TNFα (brown) and hematoxylin (DNA, blue). Scale bars = 50 µm. (**d**) *C. albicans* load in the lung of WT (C57BL/6) and dectin-1 deficient mice infected with of 3 x$10^6$ CFU of *hgc1Δ C. albicans* yeast 12 hours post infection (n=3). Statistics by unpaired t test: NS = not significant p > 0.05. (**e**) Overview of neutrophil infiltration in lungs of WT (C57BL/6) and dectin-1 deficient mice infected with of 3 x $10^6$ CFU of *hgc1Δ C. albicans* yeast. Fixation and staining of lung sections with hematoxylin and eosin (HE) 36 hours post infection. Scale bars = 1 mm. | Data are representative of two independent experiments. (a and c) Stainings courtesy of Dr Qian Wang.

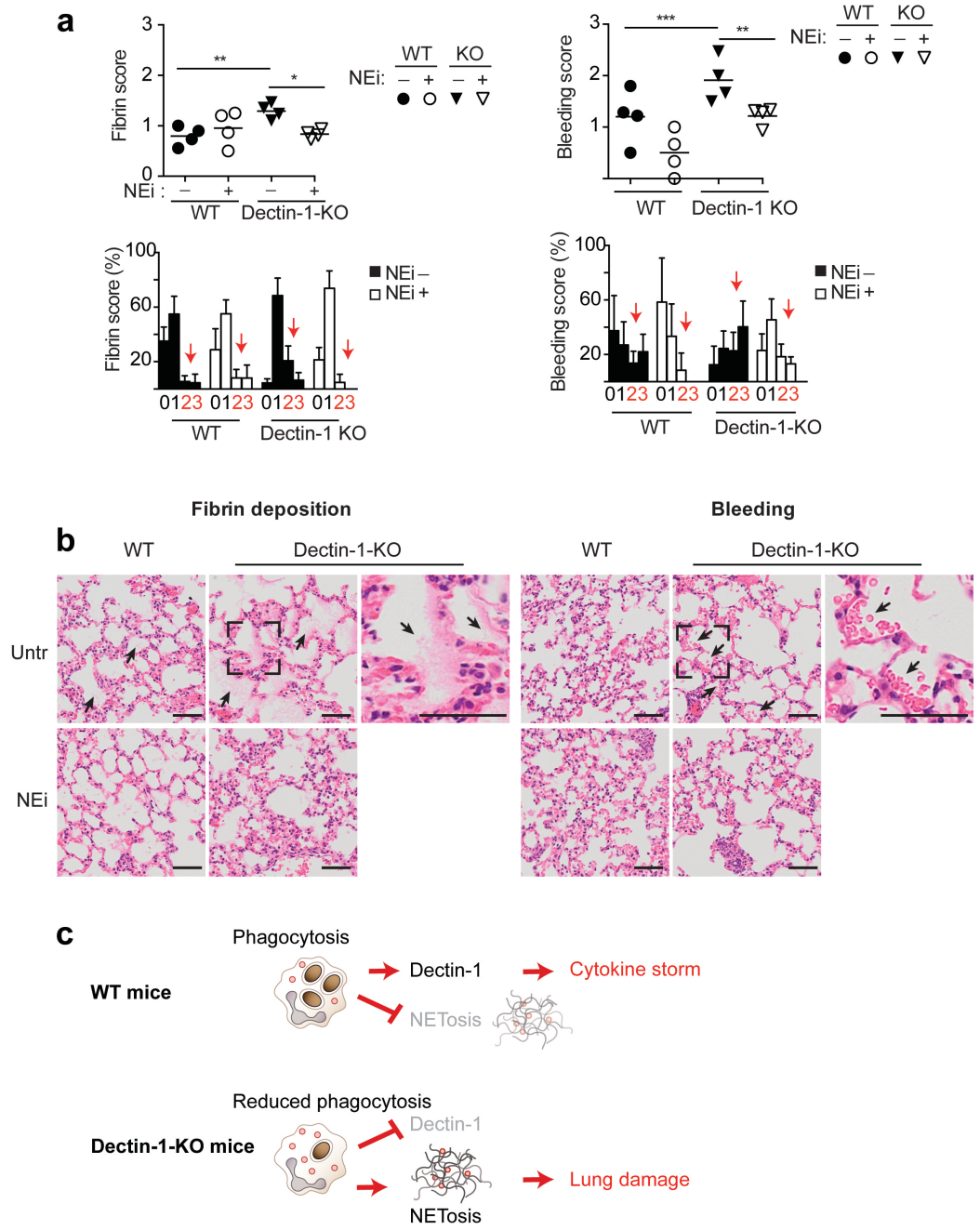


**Figure 15 | Dectin-1 KO mice show increased NET-mediated tissue damage.** (**a**) Quantification of fibrin deposition (left) and bleeding (right) in lungs of wild-type and dectin-1-deficient mice left untreated (-) or treated with 2.5 µg/ g mouse of the NE inhibitor (NEi) (+), infected with 3 × $10^6$ colony forming units (CFU) of *hgc1Δ C. albicans* yeast, assessed 36 hours later. Presented as average score for each mouse (top) or percentage of images from all mice with each score (bottom). Each symbol (top) represents an individual mouse. Statistics by two-way ANOVA followed by Tukey's multiple-comparison post-test * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$. Small horizontal lines indicate the mean. Error bars = SD, standard deviation. (**b**) Lungs of WT (C57BL/6) and dectin-1 deficient mice infected with 3 x $10^6$ CFU of *hgc1Δ C. albicans* yeast. Fixation and staining of lung sections with hematoxylin and eosin (HE) 36 hours post infection. Arrows indicate tissue damage manifested in fibrin deposition and bleeding. Right panels depict higher magnification of areas indicated by black dotted squares. Scale bars = 50 µm (**c**) Graphic overview of the causes of pathology in WT (C57BL/6) and dectin-1-deficient mice after infection with *hgc1Δ C. albicans* yeast. | (a and b) Data are representative of three independent experiments.

Taken together, we show for the first time that neutrophil antimicrobial strategies are regulated. While large microbes induce NET release, small microbes are phagocytosed. Furthermore, phagocytosis is a master regulator of NET release by sequestering NE in the phagosome and preventing translocation to the nucleus, a crucial step for NETosis. We show that NETosis is a crucial antimicrobial strategy, selectively targeted at large microbes. However, tight regulation of NETosis is crucial to prevent damage to the surrounding tissues.

## 2.6 Investigation of upstream NET-triggering mechanisms

The following chapter contains preliminary data and represents work in progress. The state of the experiments and outlook onto future work is indicated in the respective sections.

### 2.6.1 Background and aims

The molecular mechanisms underlying NET release are partially understood (Metzler, 2014; Papayannopoulos, 2010). However, little is known about the initial activators of NET release. ROS triggers NETosis (Fuchs, 2007; Palmer, 2012). Furthermore, PMA directly activates protein kinase C (PKC) and induces ROS and NETosis (Fuchs, 2007). However, these stimuli bypass the initial activation steps triggered by complex microbial particles. It is unknown which steps lie upstream of these pathways and which receptors activate them. It is likely that different microbes engage different receptors, leading to different pathways that finally converge into one NETosis activation program. This concept is also underlined by the fact that direct activation of PKC by PMA triggers NETosis in 60-80% of human neutrophils *in vitro*, whereas only 10-20% of neutrophils release NETs upon stimulation with *C. albicans* hyphae. These findings indicate that microbe-specific activation steps exist.

That only a specific percentage of neutrophils undergo NETosis suggests that there may be subsets of neutrophils that respond differentially to microbial activation. These neutrophil subsets could differ in their activation states. Since priming leads to increased activation and a potentiated antimicrobial response in neutrophils (Condliffe, 1998), pre-activation could be required for the induction of the NET pathway. Accordingly, provision of priming signals could increase the percentage of NET-releasing neutrophils. Interestingly, a recent study suggests differences in neutrophil activation and response depending on ageing. Aged $CD62L^{lo}$ neutrophils are more activated and are characterised by increased NET release compared to their $CD62L^{hi}$

counterparts (Zhang, 2015). Furthermore, neutrophil populations could differ in their surface receptor expression. Many PRRs involved in *C. albicans* recognition are known but it is unclear whether these receptors are relevant for NET activation. Receptor expression could in turn be dependent on priming and a defined activation state of these neutrophils. Priming with GM-CSF has been described to upregulate expression of TLR2, TLR4 and TLR9 in neutrophils (Hayashi, 2003; O'Mahony, 2008). However, different neutrophil populations based on the expression of PRRs have not been described so far.

Therefore, we asked:

Does priming enhance the capacity of neutrophils to respond by NETosis upon stimulation with *C. albicans*?

Which PRRs are required for *C. albicans*-mediated initiation of the NETosis pathway? Which *C. albicans* ligands are necessary for NET induction?

### 2.6.2 Priming of neutrophils does not influence NET release

Priming of neutrophils with GM-CSF has been shown to increase the neutrophil fungicidal activity. A study indicated that GM-CSF produced by NK cells is required for the activation of neutrophils and their capacity to raise a sufficient antifungal immune response (Bar, 2014; Whitney, 2014). However, it is unknown which neutrophil antimicrobial strategy is influenced by GM-CSF priming. Therefore, we investigated whether GM-CSF had an activating effect, leading to an increased percentage of NETing neutrophils.

We treated neutrophils with GM-CSF prior to stimulation with *C. albicans*. In our hands no positive effect of GM-CSF priming on the population of NETing neutrophils was observed. On the contrary, in some experiments NET release was slightly decreased (**Figure 16a**), whereas in others no difference to the un-primed controls was seen (data not shown). Interestingly, it has been shown that GM-CSF upregulates expression of dectin-1 in macrophages

(Willment, 2003), suggesting that decreased NET release could be a consequence of increased phagocytosis.

During infection, IL-8 mediates the release of neutrophils from the bone marrow into the tissues. Through priming with IL-8 neutrophils adopt an activated phenotype that equips them for antimicrobial action (Roberts, 1993; Zeilhofer and Schorr, 2000). Therefore, we investigated whether IL-8 would increase NETosis upon stimulation with *C. albicans*. However, no effect of IL-8 priming was seen upon stimulation with *C. albicans* as compared to untreated controls (**Figure 16b**).

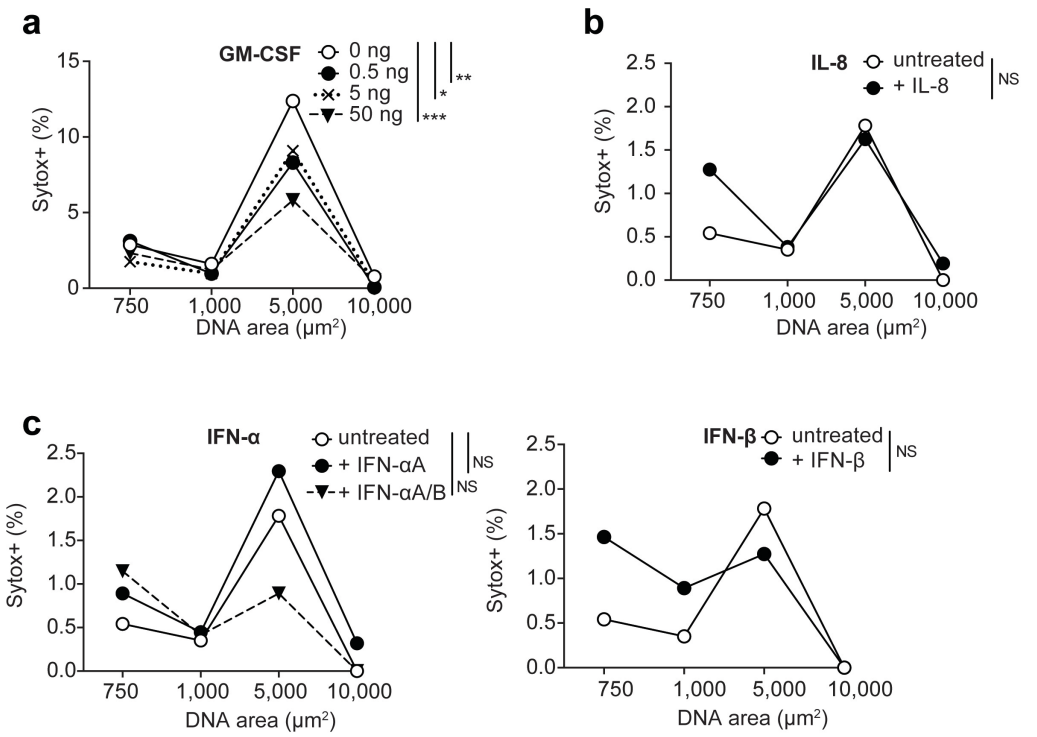


**Figure 16 | Priming of neutrophils does not influence NET release.** (**a** - **c**) NET release by human peripheral neutrophils pre-treated with (a) indicated amounts of GM-CSF, (b) 5 ng/ml IL-8 or (c) 500 U/ml IFN-α (left) or INF-β (right) prior to stimulation with *C. albicans* wild-type hyphae. NET release presented as SYTOX+ events relative to total neutrophils. | Statistics by one-way ANOVA followed by Dunnett's multiple comparison post-test. NS = not significant $p > 0.05$, *$p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Data are representative of at least three independent experiments.

It has been reported that priming neutrophils with INF-α leads to increased NET release upon subsequent complement component 5a (C5a) stimulation (Martinelli, 2004). In SLE, NETs activate pDCs to secrete IFN-α, which in turn triggers further NETosis (Garcia-Romo, 2011). Interestingly, in our hands type I interferon did not yield increased numbers of NETing neutrophils as compared to untreated controls (**Figure 16c**).

Overall, priming of neutrophils did not have a specific effect on *C. albicans*-induced NET release.

### 2.6.3 Neutrophil receptors in NET release

#### *TLR signalling in C. albicans-induced NETosis*

A variety of PRRs recognise *C. albicans*, including CLRs and TLRs (Netea, 2015a; Romani, 2011). (See introduction **Innate anti-fungal immunity** on page 77.) However, it is unknown whether these receptors are required to for *C. albicans* to trigger NETosis. We found that phagocytic receptors such as dectin-1, CR3 and FcγR downregulate NETosis, rather than being involved in the initiating signalling events (Branzk, 2014) (See results **Figure 9**.) Therefore, we screened most TLRs known to be involved in *C. albicans* signalling, using blocking antibodies.

Blocking TLR1, TLR2, TLR4 or TLR6 individually or in combination yielded no significant decrease in NET release upon stimulation with *C. albicans.* Similarly, blocking the TLR4 co-receptor CD14 alone or in combination with TLR4 had no effect on NET release (**Figure 17a**). TLR4 blockade alone was more variable depending on the donor (data not shown), but overall did not exhibit a significant effect on NET release.

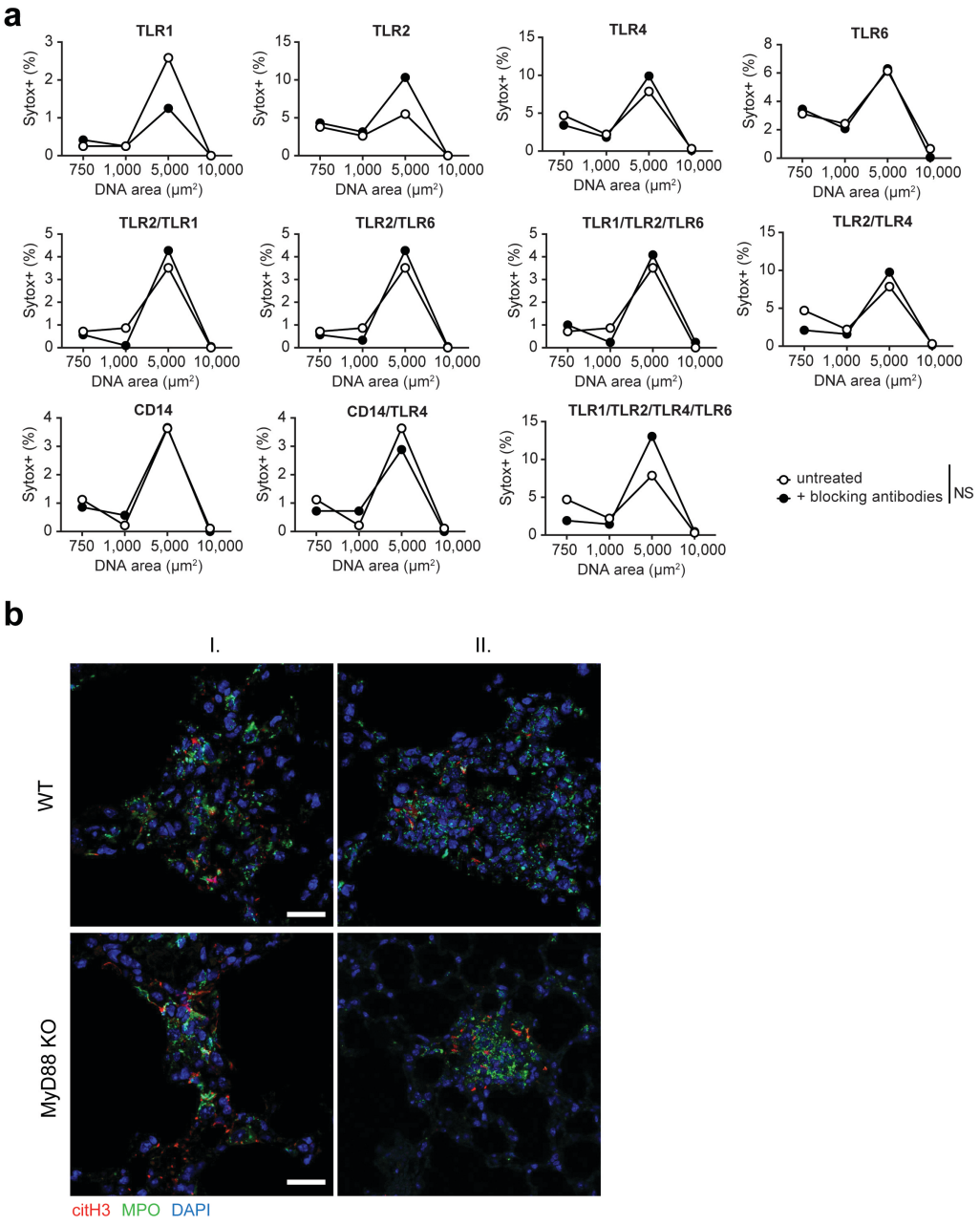


**Figure 17 | TLR signalling in *C. albicans*-induced NETosis.** (**a**) NET release by human peripheral neutrophils pre-treated with 10 µg/ml of the indicated anti-TLR blocking antibodies prior to stimulation with *C. albicans* pre-formed wild-type hyphae. NET release presented as $SYTOX^{+}$ events relative to total neutrophils. Statistics by one-way ANOVA followed by Dunnett's multiple comparison post-test. NS = not significant $p > 0.05$. Data are representative of at least three independent experiments. (**b**) NET release in the lungs of wild-type (C57BL/6) or MyD88 KO mice 24 hours after intratracheal infection with $1 \times 10^5$ colony-forming units (CFU) of *C. albicans* pre-formed wild-type hyphae, assessed by immunofluorescence microscopy of citrullinated histone H3 (citH3; red), MPO (green) and DNA (DAPI, blue). Representative images of two animals (I. and II.). Scale bars = 20 µm.

In these experiments, results were variable between different repeats with differences in observed NETosis depending on the neutrophil donors (data not shown). Donor variability is a problem in this type of experiments since heterogeneity in receptor expression between individuals is likely. Interestingly, we also observed differences between the same donors on different dates of blood donation (data not shown). This could potentially be explained by different immune states within individual donors due to illness or life style-related factors. Furthermore, although the blocking specificity and efficiency of the antibodies had been determined in previous studies, they remain to be validated in this experimental set-up.

MyD88 is the main signalling adaptor for TLRs in neutrophils. Therefore, we tested whether MyD88 deficiency influenced NET release. We infected MyD88-deficient mice with *C. albicans* pre-formed hyphae intra-tracheally. MyD88 KO mice exhibited a NET response similar to the WT controls, as indicated by comparable levels of citrullinated histone 3 (citH3) in the lungs of both groups of animals (**Figure 17b**). Therefore, MyD88-mediated signalling is not involved in triggering NET release upon stimulation with *C. albicans* in mice.

### *Kinase signalling involved in C. albicans-induced NETosis*

Different signalling pathways are involved in *C. albicans* recognition, including CLR signalling via Syk (Netea, 2015a). Furthermore, signalling via the Raf-MEK-ERK pathway was described in PMA-stimulated NET formation (Hakkim, 2011). Therefore, we used inhibitors of different candidate kinases to block pathways that were involved in fungal recognition or NET formation.

We obtained varying results depending on the neutrophil donor similar to the receptor blocking approach (data not shown). However, taken together neither inhibition of Syk, PI3K, PKC, MEK nor Jak influenced NET release significantly. Using this system, we could not identify any kinase directly involved in triggering NETosis upon stimulation with *C. albicans* (**Figure 18**).

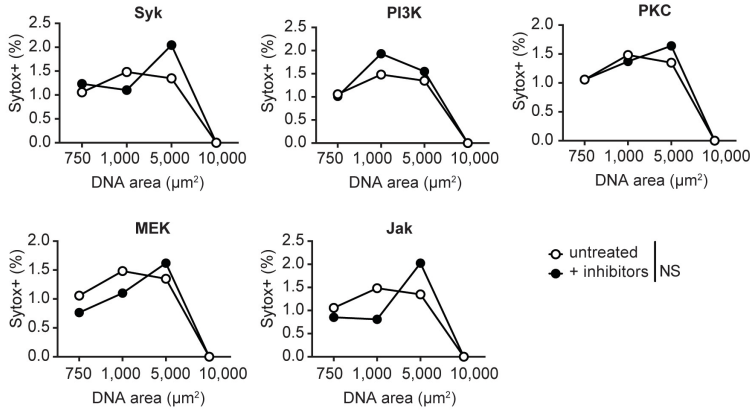


**Figure 18 | Kinase signalling in *C. albicans*-induced NET release.** NET release by human peripheral neutrophils pre-treated with inhibitors of the kinases Syk (BAY 61-3606; 1 µM), PI3K (LY294002; 2 µM), PKC (Gö 7874; 1 µM), MEK (PD184352; 2 µM) or Jak (Ruxolitinib; 0.5 µM). prior to stimulation with *C. albicans* pre-formed wild-type hyphae. NET release presented as $SYTOX^{+}$ events relative to total neutrophils. Statistics by one-way ANOVA followed by Dunnett's multiple comparison post-test. NS = not significant $p > 0.05$. Data are representative of at least three independent experiments.

## *The capacity of C. albicans cell wall mutants to trigger NETosis in vitro*

The *C. albicans* cell wall is a complex structure build up of different layers (Netea, 2008). The architecture of the cell wall is established by a variety of fungal enzymes, including mannosylases that attach mannose residues to cell wall proteins in a glycosylation reaction. Modified cell wall proteins are the main PAMPs recognised by the innate PRRs. Therefore, we investigated whether distinct cell wall components were specifically required for neutrophil recognition and triggering of NET release.

We employed *C. albicans* strains with mutations in different mannosylases (Netea, 2006). The mutants NGY 357 and NGY 600 were deficient in the enzymes och1 and [mnn2]6 respectively. These enzymes were responsible for attaching mannose residues to the cell wall proteins in an N- linked manner. The mutant NGY 337 was deficient in the enzymes mnt1 and mnt2, which were responsible for O-linked mannosylation. NGY 355 and CDH15 carried mutations in enzymes responsible for production of phosphomannan. We tested these mutants for their capacity to induce NET release *in vitro*.

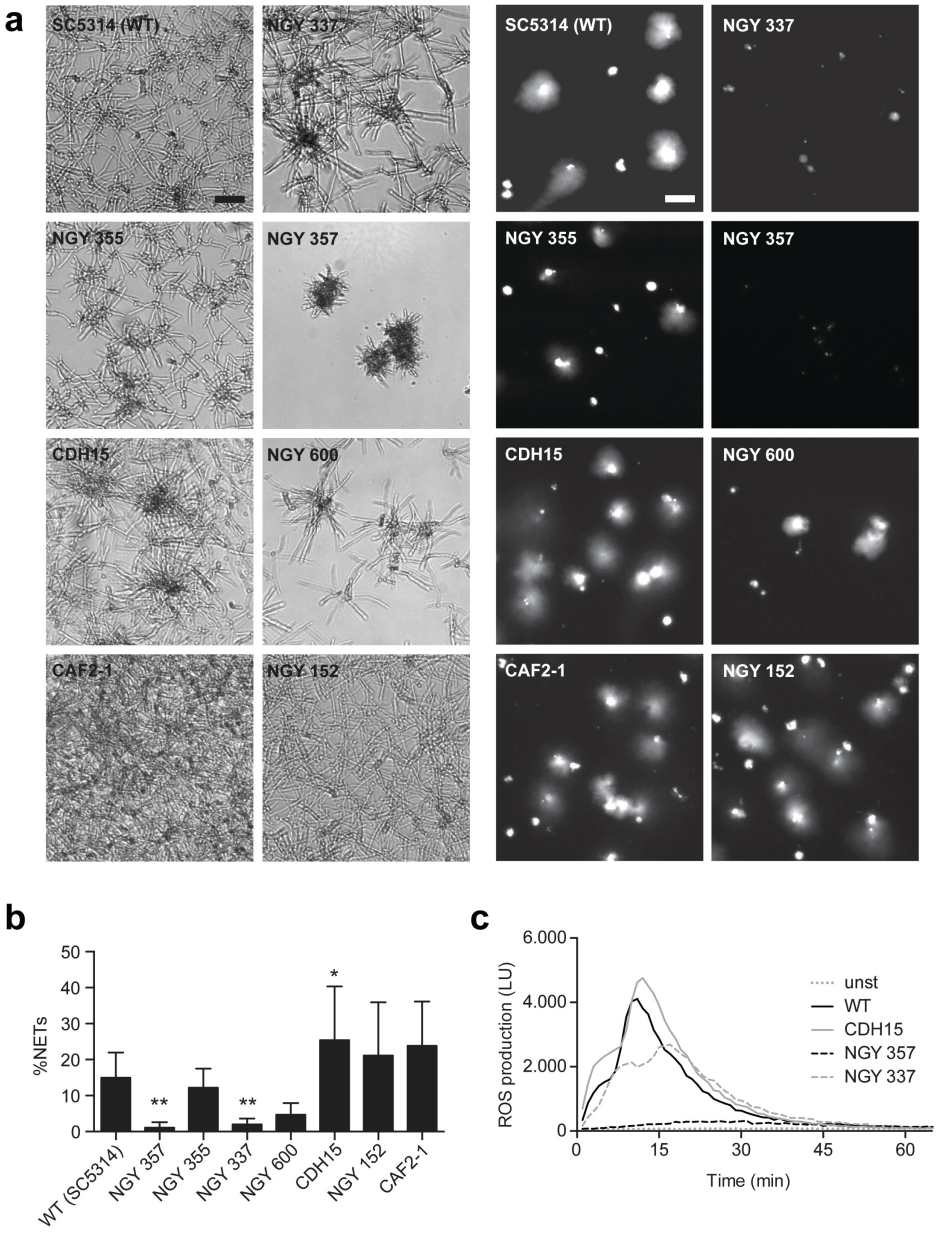


**Figure 19 | Capacity of *C. albicans* cell wall mutants to trigger NETosis *in vitro*.** (**a**) Release of NETs by human peripheral neutrophils stimulated with wild-type (WT) or mutant *C. albicans* pre-formed hyphae, MOI = 10. Extracellular DNA was stained with SYTOX 4 hours after stimulation (right). Brightfield microscopy of the neutrophils and *C. albicans* (left). Scale bars = 50 µm. (**b**) Quantification of NET release in (a) represented as %NETs of total $SYTOX^{+}$ events. Statistics by one-way ANOVA followed by Dunnett's multiple comparison post-test. Where not otherwise indicated: not significant (NS) $p > 0.05$; otherwise *$p \leq 0.05$ and ** $p \leq 0.01$ compared to WT (SC5314). Error bars = SD, standard deviation. (**c**) Production of reactive oxygen species (ROS) by human peripheral neutrophils stimulated with WT or mutant *C. albicans* pre-formed hyphae, MOI = 10. LU, luminescence units. | Data are representative of at least three independent experiments.

We stimulated human neutrophils with the hyphae of all *C. albicans* cell wall mutant strains. Interestingly, hyphae of the NGY 357, NGY 600 and NGY 337 mutant strains showed greatly decreased NET release compared to WT (SC5314) *C. albicans* hyphae and the control strains NGY 152 and CAF2-1. NGY 335 and CDH15 mutants did not show impaired NET release (**Figure 19a** and **b**).

Since reactive oxygen species are a hallmark of NET release, we investigated the capacity of the "NET-deficient" mutants to induce ROS production in neutrophils. Interestingly, neutrophils stimulated with these mutants showed significantly decreased levels of ROS production (**Figure 19c**).

Taken together, these results indicate that N- and O-linked mannosylation on *C. albicans* is required for sufficient activation of NETosis *in vitro*.

### *The capacity of C. albicans cell wall mutants to trigger NETosis in vivo*

We identified three *C. albicans* cell wall mutants that had lost their capacity to trigger NET release in human neutrophils *in vitro*. Next, we investigated their capacity to induce NETosis in a lung infection model in mouse.

Surprisingly, the mutants NGY 357 and NGY 337 that lacked NET-inducing capacity *in vitro* induced NET release in the lungs of infected mice. NET induction of NGY 357 was comparable to WT *C. albicans*, whereas NGY 337 NET induction was only slightly reduced. Interestingly, the phosphomannan-deficient strain CDH15 that had induced NETosis in human neutrophils *in vitro*, did not trigger NET release in the *in vivo* infection. This was indicated by greatly reduced levels of citrullinated histone 3 in the lung (**Figure 20**).

Differences in cell surface receptors between mouse and human neutrophils may account for the difference in the response towards these *C. albicans* strains. Otherwise, neutrophils derived from peripheral blood may behave differently to alveolar neutrophils. Furthermore, effects of other cell types

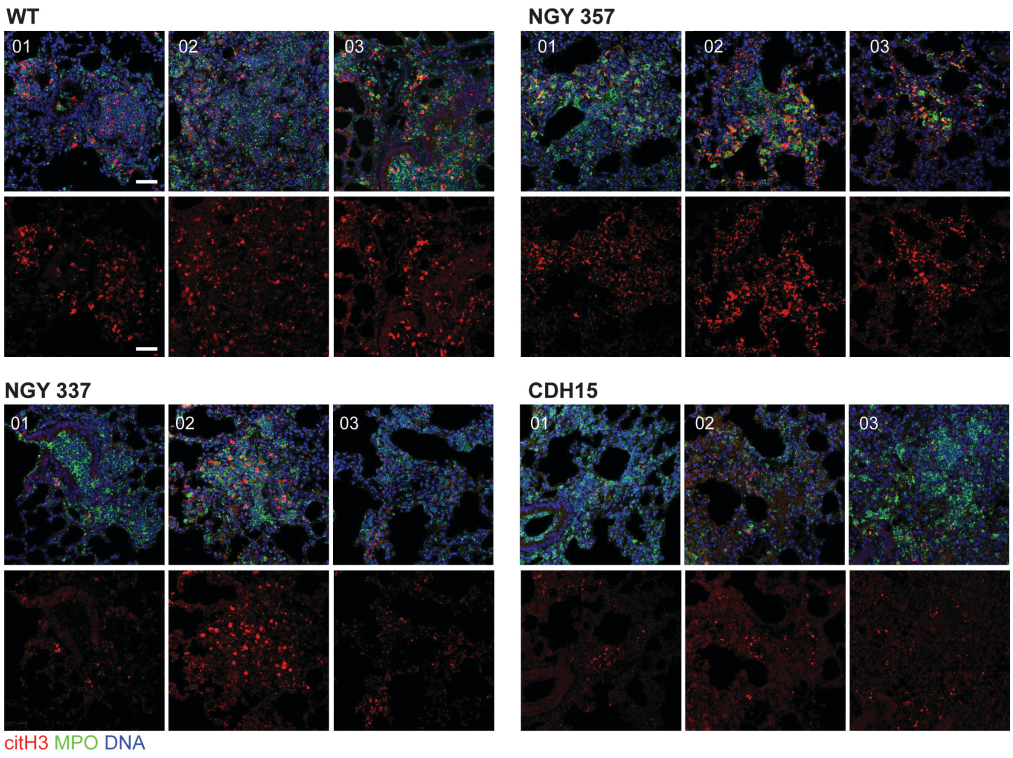


**Figure 20 | Capacity of *C. albicans* cell wall mutants to trigger NETosis *in vivo*.** NET release in the lungs of wild-type (C57BL/6) mice 24 hours after intratracheal infection with 1 × $10^5$ colony-forming units (CFU) of WT and mutant *C. albicans* pre-formed hyphae, assessed by immunofluorescence microscopy of citrullinated histone H3 (citH3; red), MPO (green) and DAPI-stained DNA (blue). Representative images of three animals (01-03). Scale bars = 50 µm.

that are provided in the tissue environment during *in vivo* infection might be lacking *in vitro*. However, we were primarily interested in neutrophil intrinsic effects and will not further investigate the interactions with other cell types.

## *Role of TLR4 and MR in C. albicans-mediated NET induction*

Our results indicated that O- as well as N-linked glycosylation of the *C. albicans* cell wall was required for sufficient induction of NET release. *C. albicans* mutants lacking these modifications failed to induce NETosis. Interestingly, a recent study indicated that both N- and O-linked

glycosylation on the *C. albicans* cell wall were necessary to trigger a sufficient pro-inflammatory immune response in macrophages. Furthermore, in macrophages TLR4 and the mannose receptor (MR) were identified to be responsible for the recognition of O- and N-linked mannans respectively (Netea, 2006). Therefore, we investigated the importance of TLR4 and MR for NET release, using blocking antibodies.

Blocking TLR4 or MR individually led to a marked decrease in NET induction upon stimulation with *C. albicans* WT in some experiments. NETosis was further decreased when TLR4 and MR were blocked in combination (**Figure 21a**). Blocking TLR4 and MR did not have any effect on NET release upon stimulation with the *C. albicans* mutants NGY 337 and NGY 357, deficient in O- and N-linked glycosylation respectively (**Figure 21a**). Importantly, these results were difficult to reproduce using neutrophils obtained from donors at different times (**Figure 17a** and data not shown) and these preliminary experiments need further verification.

Next, we tested whether signalling via MR or TLR4 was required for NET induction. Therefore, we used saturating amounts of soluble mannan and LPS in competitive blocking experiments. Indeed, pre-incubation of neutrophils with high concentrations of LPS and Mannan decreased NETosis upon stimulation with WT *C. albicans* hyphae in a dose-dependent manner compared to unblocked neutrophils (**Figure 21b**). Again, due to donor variability that was observed between experiments (data not shown), these experiments need further verification.

Next, we investigated whether MR and TLR4 were required to trigger production of ROS. Interestingly, *C. albicans*-induced ROS production was decreased in the presence of anti-MR and anti-TLR4 blocking antibodies (**Figure 21c**). Furthermore, competition with high doses of Mannan and LPS decreased ROS production upon stimulation with *C. albicans* hyphae (**Figure 21d**).

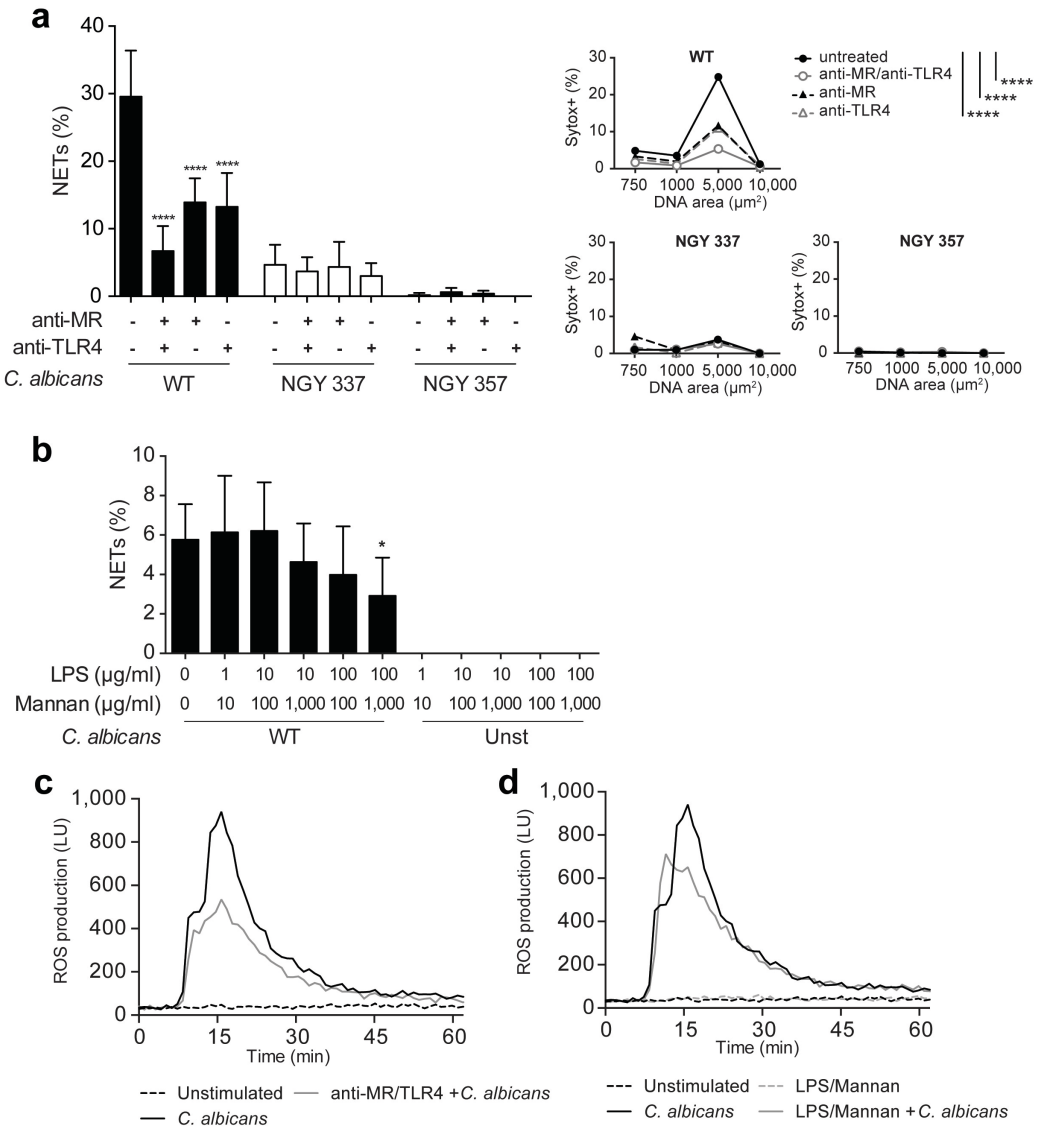


**Figure 21 | Role of TLR4 and MR in *C. albicans*-mediated NETosis.** (**a**) NET release by human peripheral neutrophils pre-treated with 10 µg/ml anti-TLR4 and anti-MR blocking antibodies prior to stimulation with wild type or mutant *C. albicans* pre-formed hyphae. NET release presented as %NETs of total SYTOX$^+$ events (left) and SYTOX$^+$ events relative to total neutrophils (right). (**b**) NET release by human peripheral neutrophils pre-treated with indicated concentrations of LPS and mannan, left unstimulated (Unst) or stimulated with *C. albicans* pre-formed wild-type hyphae. (**c**) and (**d**) Production of reactive oxygen species (ROS) by human peripheral neutrophils pre-treated with 10 µg/ml anti-TLR4 and anti-MR blocking antibodies (c) or pre-treated with 100 µg/ml LPS and 1000 µg/ml mannan prior to stimulation with WT *C. albicans* pre-formed hyphae. LU, luminescence units. | MOI = 10. Statistics by one-way ANOVA followed by Dunnett's multiple comparison post-test. Where not otherwise indicated: not significant (NS) $p > 0.05$; otherwise *$p \leq 0.05$, **** $p \leq 0.0001$. Data are representative of at least three independent experiments. Error bars = SD, standard deviation.

These preliminary data suggest that MR and TLR4 might play a role in the *C. albicans*-mediated onset of NETosis. Notably, these results need further careful validation. Furthermore, it is not known which ligand on *C. albicans* would be recognised by TLR4. Even though there are descriptions of fungal lipopolysaccharide (Cheng, 2005), most fungi, including *C. albicans* do not contain LPS. Furthermore, previous studies claiming that fungi activate TLR4 (Meier, 2003; Netea, 2003; Tada, 2002) need to be interpreted with caution. Possible contamination with the ubiquitous bacterial LPS in these studies cannot be completely excluded.

Taken together these data show that N- and O- linked mannosylation of *C. albicans* cell wall proteins is required for the activation of the NETosis pathway. Since previous studies have linked MR and TLR4 to *C. albicans* recognition, our findings may indicate that MR and TLR4 are responsible for the recognition of these glycosylated structures on *C. albicans* and required for NET induction. However, the results need to be validated carefully since great donor variation between the experiment occurred.

# *3 DISCUSSION*

Neutrophils control efficiently a large variety of microbes. However, until now they were thought to carry out a single defence program with little capacity to adapt. It was unknown whether neutrophils were able to regulate their antimicrobial strategies and selectively target individual pathogens. Strikingly, in this study we found that neutrophils are able to regulate their antimicrobial strategies and fine-tune their response according to the type of microbe they encounter. We uncovered a novel mechanism that regulates the release of NETs by allowing neutrophils to sense the size of a microbe based on their capacity to phagocytose the particle. We show that phagocytic receptors such as dectin-1 are important for sensing and regulating the immune response.

Our data demonstrate the importance of microbe size in modulating innate immune effector strategies. Previous findings show that immune receptors can discriminate between soluble and particulate ligands and are able to trigger varied responses accordingly. Innate immune cells such as macrophages and neutrophils sense β-glucan through dectin-1 and can discriminate whether receptor activation occurred in the context of a particulate microbe or by soluble β-glucan shed from distant microbes (Goodridge, 2011). This discrimination is important for the initiation of an adequate immune response, which eliminates microbes locally while preventing unnecessary inflammatory responses. In this context particulate β-glucan cross-links dectin-1 in synapse-like structures and drives the exclusion of regulatory tyrosine phosphatases, which initiates downstream Syk signalling. Soluble β-glucan fails to exclude the inhibitory phosphatases, leading to suppression of dectin-1 signalling. Furthermore, in dendritic cells frustrated phagocytosis prolongs dectin-1-signalling and increases kinase activation, leading to elevated levels of pro-inflammatory cytokines *in vitro* (Hernanz-Falcón, 2009). Another study suggests that *C. albicans* yeast-mediated dectin-1 ligation on DCs and subsequent release of IL-6 leads to the induction of a protective Th17 response in the skin (Kashem, 2015a). In contrast, invasive *C. albicans* hyphae induce a Th1 response, due to their lack

of β-glucan exposure and failure to activate dectin-1. These findings indicate a regulatory function of dectin-1 signalling in DCs.

In our results we did not observe significant differences in kinase activation in response to particles of different size. Phosphorylation of Syk and ERK kinases was prominent in response to small yeast and large hyphae. These results show that that exclusion of regulatory phosphatases or prolonged dectin-1 signalling does not play a role in regulating the antimicrobial response towards microbes of varying size, including the different *C. albicans* growth forms, in neutrophils.

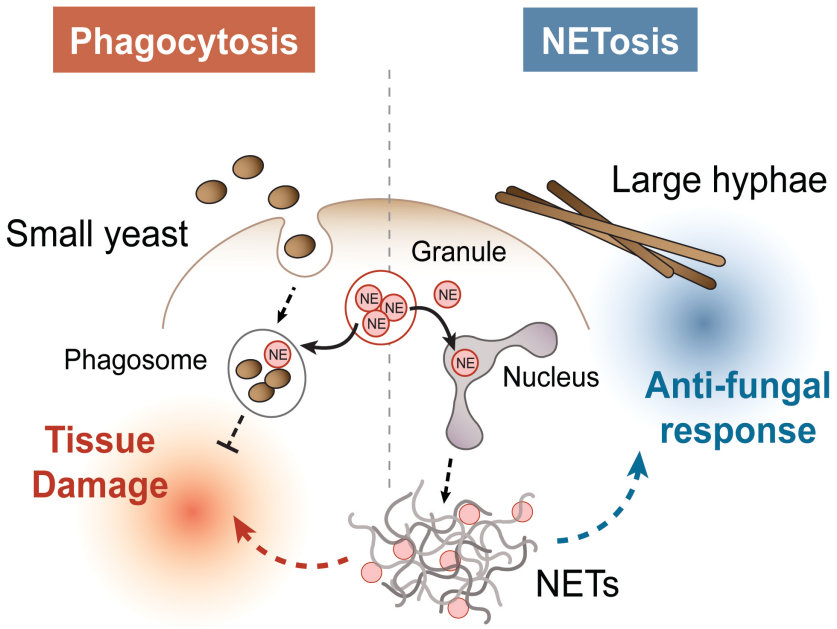


**Figure 22 | Phagocytosis negatively regulates NETosis.** Phagocytosed yeast particles drive the translocation of NE to the phagosome via fusion with azurophilic granules, sequestering NE away from the nucleus. In contrast, during the response to hyphae in the absence of a phagosome, NE translocates from azurophilic granules to the nucleus, processing histones to drive chromatin decondensation. By inhibiting NETosis, phagocytosis prevents tissue damage caused by uncontrolled NET release.

In contrast, we show that selective NET release is based on the competition between phagocytosis and NETosis for the availability of NE (**Figure 22**). Phagocytosis is a rapid event in neutrophils, leading to uptake of particles and formation of phagosomes within minutes. Phagosome maturation in neutrophils requires the fusion of the phagocytic vacuole with granules, depositing granule content in the phagosome. The fusion step sequesters NE in the phagosome and depletes the general cellular pool of this molecule. However, translocation of NE into the nucleus is an absolute requirement for NETosis. NE processes histones to decondense chromatin, leading to NET release. Here we show that phagocytosis is a negative regulator of NETosis by depleting the cellular NE pool and preventing NE translocation into the nucleus. Inhibition of phagocytosis by physical prevention of particle uptake via a modified transwell or by blocking of the phagocytic activity of dectin-1 leads to the release of the negative regulation and increase in NETosis. Increasing numbers of phagocytosed beads, leading to increasing numbers of regulatory phagosomes caused a dose-dependent decrease of NET release upon stimulation with *C. albicans* hyphae. These findings indicate that prolonged dectin-1 signalling through frustrated phagocytosis alone is not sufficient to trigger NETosis. Instead, NET release is regulated by the fate of NE. In the case of undisturbed NE translocation, the nucleus is driven into chromatin decondensation, entirely independent of upstream dectin-1 signalling.

Our findings suggest that if a microbe is small enough to be taken up, it can be controlled intracellularly in the phagosome. However, if phagocytosis fails, an extracellular strategy needs to be in place to prevent outgrowth and dissemination of the microbe and NETosis is triggered. Our study reveals that failure to trigger NET release upon encounter with large microbes increases susceptibility and impairs clearance of infection. Patients with CGD suffer from recurrent bacterial and fungal infections. Previous studies show that reconstitution of NADPH oxidase activity and ROS production through gene therapy results in resolution of fungal infections in these patients and

restoration of NETosis *in vitro*. However, since CGD patients are also deficient in phagocytosis it is unclear which contribution NET release had in the clearance of these infections (Bianchi, 2009). In contrast, patients with full MPO deficiency are mainly susceptible to fungal infections and *in vitro* experiments show that neutrophils from MPO-deficient patients fail to form NETs (Metzler, 2011). Studies indicate that the group A *Streptococcus* (GAS) DNase Sda1 degrades NETs, representing an immune evasion strategy. Sda1-deficient GAS mutants fail to degrade NETs and are less virulent *in vivo* (Buchanan, 2006). However, since Sda1 also degrades biofilm formation, the importance of NETs in clearing infection cannot be determined entirely with these susceptible microbes. In contrast, our experiments, using MPO-KO mice allowed us to directly compare infections in NET-deficient with NET-competent mice *in vivo*. In combination with NET-inducing or non-inducing microbes such as *C. albicans* hyphae and yeast, respectively, this experimental set-up provided us with a strong tool to dissect the importance of NET release for antifungal defence *in vivo.* Here we show for the first time directly that NETosis is crucial for protection against large microbes, as NET-deficient mice succumb to fungal infection with large hyphae. These results demonstrate that selective NETosis is required for effective antifungal immunity.

We demonstrate that NETosis is an extracellular antimicrobial strategy selectively targeted at large microbes. However, there is a vast literature describing NET release upon stimulation with small bacterial stimuli. In many instances different approaches in NET detection might account for seemingly conflicting data. For instance, we did not observe NET release in response to various bacteria *in vitro* or *in vivo*. However, bacteria have developed various immune evasion strategies that include prevention of phagocytosis, phagosomal escape and degradation of NETs. The regulatory mechanism we uncovered here might ensure that NETs are released in response to virulent small microbes that disrupt phagocytosis. For example, phagosome lysis would liberate NE to translocate to the nucleus and promote

NET formation. Alternatively, we demonstrate that *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) forms large aggregates, which cannot be taken up via phagocytosis induce NETosis, whereas single BCG bacteria are phagocytosed and do not trigger NET release. Hence, sensing bacterial aggregation to release NETs might be important in controlling large bacterial aggregates and critical in defence against biofilms and abscess formation. Interestingly, BCG is able to disrupt phagosome maturation, but this mechanism was not relevant in driving NETosis since the small, phagocytosed bacteria failed to induce NETs in our experiments. Yet, NET release in response to microbial interference with phagosome maturation might be relevant in response to other bacteria. Previous studies describe that *Neisseria gonorrhoea* evades intracellular killing by delaying fusion of the phagosome with primary and azurophilic granules (Johnson and Criss, 2013). This delayed fusion opens up a time window in which sufficient amounts of NE could translocate to the nucleus to induce NETosis. Accordingly, *N. gonorrhoea* has been shown to induce NETs (Juneau, 2015b). Indirect evidence of phagocytic regulation of NET release is also provided by different strains of the gingival bacterium *Porphyromonas gingivalis* that trigger exclusively either phagocytosis or NETosis dependent on the presence or absence of the virulence factor gingipain respectively (Jayaprakash, 2015). Whether NETosis has evolved as a back-up immune strategy to capture small microbes that evade the first line of phagocytic killing or whether it is a microbial immune evasion strategy, employed by bacteria to deplete neutrophils and other immune cells (Thammavongsa, 2013) is not clear. In order to answer this question it needs to be clarified whether NETs are effective in controlling bacteria. Importantly, a non-lytic form of NETosis that leads to live cytoplasts that are able to migrate and phagocytose (Pilsczek, 2010; Yipp, 2012) adds an additional layer of complexity to this question.

Selective employment of the neutrophil antimicrobial strategies is not only required for sufficient antimicrobial defence but may also have a detrimental

effect on the host. NETs contain a range of antimicrobial agents and cytotoxic mediators, including potent proteases as well as histones that have been shown to cause tissue pathology (Xu, 2009). Studies in humans indicate that failure to clear NETs from the tissue environment causes damage to the organism (Hakkim, 2010). Our findings highlight the need for a tight regulation of NETosis that serves as a protective mechanism that prevents unnecessary tissue damage driven by excessive NET release. Importantly, mutation or blocking of dectin-1 decreases phagocytosis, releasing the regulatory block of NE sequestration and allowing NE translocation and NET release also upon stimulation with small fungal particles. NETosis triggered by small microbes represents a de-regulation, since these particles can be eliminated intracellularly. We demonstrate for the first time a direct link between the disregulation of NET release and tissue pathology caused by the increased NETosis. Furthermore, in agreement with our findings, recent studies show the release of NETs upon stimulation with larger particulate crystal structures such as monosodium urate or cholesterol crystals (Schorn, 2012; Warnatsch, 2015). NETs triggered by cholesterol crystals have been shown to contribute to the inflammatory phenotype of atherosclerosis by amplifying inflammasome activation (Warnatsch, 2015). Therefore, while NET release upon stimulation by large microbes mediates clearance of infection and containment of inflammation, NET activation by sterile particles leads to prolonged persistence of NETs and amplification of inflammation. These data confirm our findings that NETosis needs to be tightly regulated to ensure efficient immune defence while preventing tissue pathology. In contrast, a study suggested that aggregated NETs release proteases that degrade cytokines, reducing inflammation and pathology in gout disease (Schauer, 2014). However, the authors did not genetically interrogate the role of NETosis by using a mouse model with enhanced NET release, but injected NET aggregates into the inflamed tissue instead. The physiological relevance of these findings remains to be investigated. According to this study NETs might be beneficial or detrimental depending on their local concentration.

The importance of regulated NET release also opens new pathways for clinical understanding of NET-related conditions. Several autoimmune diseases are characterised by the presence of anti-nuclear autoantibodies. Not much is known about the triggers of autoimmune disorders such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) and the original source of the auto-antigens that cause the initial break of tolerance. The release of NETs into the extracellular space provides a source of nuclear auto-antigens, including double-stranded DNA and citrullinated histones. Understanding the regulation of NETosis could help to understand the onset of autoimmune diseases. According to our findings it is possible that people with mutations in phagocytic receptors are prone to increased NETosis. In situations of severe infection, elevated NET release could excessively expose nuclear autoantigens, causing tolerance to break. Interestingly, studies describe an increased incidence of mutations in complement receptor CR3 in patients with SLE (Zhou, 2013). These mutations cause the complement-mediated phagocytic pathway to be partially reduced. While the incidence of these mutations is around 30% in the healthy, undiagnosed population, it is increased to up to 70% incidence in SLE patients. This indicates that mutations in phagocytic receptors could be a potential risk factor for the onset of autoimmune disease caused by the loss of NET regulation.

To completely comprehend the circumstances under which NETs are released, it is important to understand the initiating signals that activate NETosis. Our results suggest that N- and O-linked glycosylation of the *C. albicans* cell wall are required for triggering NETosis and that TLR4 and MR might be involved in transducing the signals. More work needs to be done to verify these results and to uncover other receptors that are required to initiate NET release. Understanding the activators of the NETosis pathway would offer further therapeutic avenues for regulating this important antimicrobial strategy.

Overall, our findings establish that neutrophils have the ability to respond selectively to different microbial triggers. The tight regulation of NET release

is crucial for a sufficient antimicrobial defence against large pathogens as well as for the prevention of host pathology. The implications of this tight regulation for the onset of for example autoimmune diseases provides potential for further investigation.

# *4 MATERIAL AND METHODS*

## Neutrophils

### Human neutrophil isolation

Peripheral blood was collected with consent from de-identified, healthy adult volunteers according to the protocol approved by the Francis Crick ethics board according to the Human Tissue Act.

Neutrophils were freshly isolated as described elsewhere (Aga, 2002). In brief: 6 ml of heparinised whole blood was overlaid over 6 ml of pre-warmed Histopaque 1119 (Sigma, 11191) in 15 ml falcon tubes and centrifuged for 20 min at 800 x g. The plasma and PBMC layer were removed with pasteur pipettes. The neutrophil layer was washed with 10 ml Hank's Balanced Salt Solution (HBSS) without magnesium, calcium or phenol red (Thermo Scientific, SH3058801) with 0.1% donor plasma (HBSS--) and centrifuged for 10 min at 300 x g. A percoll (GE healthcare, 17-5445-02) gradient was prepared from layers of 2 ml each. The layers were from the bottom: 85%, 80%, 75%, 70% and 65%. 2 ml of neutrophils re-suspended in HBSS-- were overlaid over the gradient and centrifuged for 20 min at 800 x g. The neutrophil layer was removed and washed in 10 ml HBSS--. The neutrophil pellet was re-suspended in 2 ml HBSS--. Neutrophils were counted in a Thoma hemocytometer. For plating in cell culture dishes, neutrophils were re-suspended in HBSS with magnesium and calcium, without phenol red (HBSS++) (Thermo Scientific, SH3026801). 3% donor plasma was added were indicated.

### Mouse neutrophil isolation

Mouse femur and tibia of the hind legs were removed and bone marrow was flushed out with sterile PBS. Neutrophils were isolated using the Easysep mouse neutrophil enrichment kit (Stemcell Technologies, 19762) following the instructions. In brief: bone marrow cells were incubated with a negative selection cocktail of antibodies targeting all cells but neutrophils. Magnetic

beads were attached to the antibodies in a second step. Finally all antibody-bound cells were captured in the reaction tube using a strong magnet. Neutrophils were poured into a new tube and washed with HBSS++.

Mouse plasma was obtained by exsanguination of anesthetised mice. The collected blood was incubated for 10 min at 37°C. Plasma was separated by centrifugation. 3% plasma was used in all *in vitro* experiments.

*NET release and quantitation*

$5x10^4$ neutrophils per well were plated on the bottom of a 24-well plate in HBSS++ in the presence or absence of 3 % human plasma as indicated. Neutrophils were stimulated with the indicated stimuli. 4 h later Sytox (Invitrogen, S7020) was added and cells were analysed for NET release by fluorescent microscopy. Images were taken using Micro-Manager (Edelstein, 2014).

NET release was scored using Fiji (Schindelin, 2012) as previously described (Papayannopoulos, 2010). In brief: Fluorescence images of Sytox signal were transformed into binary images. The area of the Sytox positive events was determined. Area values were distributed into bins of increasing area sizes and the results were plotted as the area of each Sytox positive event over total number of cells in the respective bins. Events with an area over 1000 $\mu m^2$ were considered NETs.

## Microbes

### Fungal strains

| Strain name | Genotype | Reference |
|---|---|---|
| *C. albicans:* | | |
| SC5314 | WT | (Gillum, 1984) |
| yeast-locked | hgc1Δ | (Zheng, 2004) |
| NGY 337 | mnt1/mnt2Δ | (Mora-Montes, 2010) |
| NGY 355 | pmr1Δ | (Bates, 2005) |
| NGY 357 | och1Δ | (Bates, 2006) |
| NGY 600 | [mnn2]6Δ | (Hall, 2013) |
| CDH15 | mnn4Δ | (Hobson, 2004) |
| NGY 152 | WT (CAI4, URA3Δ, isogenic to SC5314) | (Brand, 2004) |
| CAF2-1 | WT (URA3 het, isogenic to SC5314) | (Fonzi and Irwin, 1993) |
| *A. fumigatus:* | | |
| NIH 5233 (ATCC 13073) | WT | (ATCC, 1307) |

### Fungal culture and hyphal preparation

All fungal strains were cultured overnight shaking in yeast extract-peptone-dextrose (YEPD) medium at 37°C and subcultured in YEPD medium the next morning. To induce hyphal growth overnight cultures were subcultured for 4 h in Roswell Park Memorial Institute (RPMI) medium.

Heat-inactivation was achieved by incubating *C. albicans* shaking for 1 h at 90°C. Hyphae were fragmented using the EmulsiFlex-C5 high-pressure homogenizer (Avestin).

*Bacterial strains*

| Strain | Reference |
|---|---|
| *E. coli* DH5α | (Laboratories, 1986) |
| *K. pneumoniae* KP52145 | (Benghezal, 2007) |
| *M. bovis* BCG-dsRed | (Kasmapour, 2012). |
| *S. pneumoniae* D39 | Dr M. Coles, University of York |

*Bacterial culture and BCG preparation*

*E. coli* and *K. pneumoniae* were cultured overnight in Lysogeny broth (LB) shaking at 37°C and subcultured the next morning to an $OD_{600}$ of 1.

*S. pneumoniae* D39 was grown in brain–heart infusion broth under microaerophilic conditions at 37°C for 16 hours to autolytic phase, then subcultured and grown to an $OD_{600}$ of 0.4, centrifuged and resuspended in PBS immediately prior to infection.

BCG-dsRed (Kasmapour, 2012) was grown to an $OD_{600}$ of 0.8 in Middlebrook 7H9 medium supplemented with 10% Oleic acid, Albumin, Dextrose, Catalase (OADC), 0.05% Tween-80, 0.4% glycerol and 50ug/ml Hygromycin at 37°C with shaking at 100 rpm. Bacterial cultures were centrifuged and the supernatant was repeatedly passed through a syringe to remove large aggregates. The obtained single cells and small aggregates were used for experiments.

## *In vitro assays*

*Immunoblotting*

$1x10^6$ human peripheral neutrophils were plated HBSS++ in 12-well plates. Neutrophils were stimulated with *C. albicans* yeast or pre-formed hyphae. At indicated times cells were lysed in Sodium dodecyl sulphate (SDS) sample buffer and stored at -80°C. A standard immunoblotting protocol was carried

out as described elsewhere. In brief: samples were loaded onto a Criterion TGX 10-20 % Tris-glycine gel (Biorad, 5671113) and run for 20 min at 300 V. Proteins were transferred onto a PVDF membrane (Biorad, 1704157), using the Trans-Blot Turbo Transfer System (Biorad, 1704155). Membranes were blocked with 5% skim milk (Sigma, 70166) for 1 h. Primary antibodies in 5% skim milk were incubated for 1h and the membrane was washed in Tris-Buffered Saline and Tween 20 (TBST). Secondary antibodies coupled to horseradish peroxidase (HRP) in 5% skim milk were incubated for 1h and the membrane was washed again in TBST. The membrane was incubated with enhanced chemiluminescent (ECL) substrate (Thermo Fisher, 32106) and developed onto a photo film in the dark room.

Primary antibodies: H3: anti-histone H3 (pan) (Millipore, 07-690), Syk: anti-Syk (N-19) (Santa Cruz, sc-1077), pSyk: anti-phospho-Zap-70 (Tyr319)/Syk (Tyr352) (Cell Signalling, 2701s), ERK: p44/42 MAPK (Erk1/2) (Cell Signalling, 9102S), pERK: ERK1/2 (pTpY185/187) (Life Technologies, 44680G). Secondary antibody: goat anti-rabbit HRP (Thermo Scientific, 31463).

*Transwell assay*

The rims of conventional 24-well transwell inserts (0.4 µm pore size, 10 µm membrane thickness) (Corning Incorporated, 3470) were cut off to enable the transwell strainer to sit directly on the bottom of the culture dish, allowing for direct contact with the neutrophils plated in the well. For stimulation, *C. albicans* yeast was seeded in the transwell strainer. 4h later neutrophils were analysed for NET release as described above.

Nuclei of neutrophils attached to the bottom of the transwell strainer were stained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) 4 h post stimulation. The strainer was placed on a coverslide and the attached cells were imaged by inverted fluorescent microscopy.

*Reactive oxygen measurement*

$1x10^6$ neutrophils were plated in white opaque 96-well plates in 100 µl HBSS++. After settlement of cells to bottom of the well, plates were pre-incubated on ice to delay ROS reaction to facilitate measurement. 50 µl reaction mix ( 1.2 U/ml horseradish peroxidase (HRP), 100 µM luminol in PBS) was added. Neutrophils were immediately stimulated with a MOI of 2.5 ($2.5x10^6$) WT *C. albicans* (yeast or pre-formed hyphae) or *C. albicans* cell wall mutants as indicated. *C. albicans* was spun onto the neutrophils and ROS production was measured immediately as luminescence units at 425 nm, using a FLUOstar Omega plate reader (BMG Labtech).

*Phagocytosis rate and blocking phagocytosis*

$3x10^5$ human peripheral neutrophils were plated on glass bottom petri dishes (MatTEK, CCS-8) and incubated for 1 h in the presence of 10 µg/ml anti-dectin-1 blocking antibody (BD6; AbD Serotec, MCA4662) prior to stimulation. Sytox was added for detection of NET release. Neutrophils were stimulated with *C. albicans* yeast or pre-formed hyphae and NET release was examined over a time of 4 h using confocal microscopy. Time-lapse images were analysed for number of phagocytosed particles by recording the number of phagocytosed particles per cell for each frame.

$5x10^4$ neutrophils per well were plated on the bottom of a 24-well plate and pre-incubated for 1 h at 37°C in the presence of blocking antibodies against mannose receptor (MR) (15-2; Invivogen, mab-hmr; 10 µg/ml), FcγR (CD16/CD32 Fc block, 2.4G2; BD Bioscience, 553142; 0.5 µg/ml) or complement receptor 3 (CR3, CD11b) (ICRF44; Biolegend, 301330; 10 µg/ml). Subsequently neutrophils were stimulated with *C. albicans* pre-formed hyphae (MOI = 10). NET release was assessed 4 h later as described above.

*Inhibition of vesicle fusion*

$5x10^4$ neutrophils per well were plated on the bottom of a 24-well plate and pre-incubated for 1 h at 37°C in the presence of 1 µM bafilomycin (Sigma, B1793) or 2.5 µM nocodazole (Sigma, M1404). Subsequently neutrophils were stimulated with *hgc1Δ* yeast-locked *C. albicans* (MOI = 10). NET release was assessed 4 h later as described above.

*Phagocytosis of yeast or beads to inhibit NETosis*

Small polystyrene beads (3 µm diameter, Krisker biotech) were opsonized for 30 min at 37°C with 100% plasma prior to stimulation. $5x10^4$ neutrophils per well were plated on the bottom of a 24-well plate and pre-incubated for 30 min at 37°C with *hgc1Δ* yeast-locked *C. albicans* or polystyrene beads to induce phagocytosis. Afterwards neutrophils were stimulated with pre-formed *C. albicans* hyphae (MOI = 10). NET release was assessed 4 h later as described above.

*Neutrophil priming*

$5x10^4$ neutrophils per well were plated on the bottom of a 24-well plate and pre-incubated for 1 h at 37°C in the presence of 0.5, 5 or 50 ng/ml recombinant GM-CSF (Peprotech, 300-03), 5 ng/ml IL-8 (R&D, 208-IL-010), 500 U/ml IFN-αA (Gibco, PHC4014), 500 U/ml IFN-αA/B (Miltenyi) or 500 U/ml IFN-β1a (Gibco, PHC4244).

*Receptor blocking*

$5x10^4$ neutrophils per well were plated on the bottom of a 24-well plate and pre-incubated for 1 h at 37°C in the presence of 10 µg/ml blocking antibodies against TLR1 (H2G2; Invivogen, mabg-htlr1), TLR2 (TL2.1; e-Bioscience, 16-9922), TLR4 (HTA125; Santa Cruz, sc-13593), TLR6 (C5C8; Invivogen, mabg-htlr6) and CD14 (HCD14; Biolegend, 325605) alone or in the indicated combinations. Subsequently neutrophils were stimulated with *C. albicans*

pre-formed hyphae (MOI = 10). NET release was assessed 4 h later as described above.

*Kinase inhibition*

5x104 neutrophils per well were plated on the bottom of a 24-well plate and pre-incubated for 1 h at 37°C in the presence of inhibitors of the kinases Syk (BAY 61-3606; Merck, 574717; 1 µM), PI3K (LY294002; Cell Signalling, 9901; 2 µM), PKC (Gö 7874; Merck, 365252; 1 µM), MEK (PD184352; Sigma, PZ0181; 2 µM) or Jak (Ruxolitinib; Invivogen, tlrl-rux; 0.5 µM). Subsequently neutrophils were stimulated with *C. albicans* pre-formed hyphae (MOI = 10). NET release was assessed 4 h later as described above.

*XTT viability assay*

Increasing amounts of neutrophils ($5x10^3$ – $5x10^5$) were seeded in 200 µl HBSS ++ in a 96-well plate. Subsequently neutrophils were stimulated with50 µl $5x10^4$ *C. albicans* pre-formed WT hyphae (MOI = 10 – 0.1 respectively). At indicated time points 50 µl of 6 mg/ml 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) was added. The plate was incubated for 30 min at 37°C to allow for the colour reaction to take place. 100 µl of the reaction were transferred in duplicates into a new 96-well plate and absorbance was read at 450 nm. Viability was expressed as fold change compared to *C. albicans* hyphae alone.

## *In vivo mouse infection*

All mouse experiments conformed to the guidelines of the UK home office under an approved project license**.**

*Mice*

The mice used in the experiments were all between 6 and 8 weeks old and sex matched. C57BL/6 mice were used as WT controls. All genetically

modified mice were bread on this background. Dectin-1 KO mice were obtained from Prof Gordon Brown (Aberdeen, UK). NADPH oxidase KO (Cybb/J) mice were obtained from Prof Arturo Zychlinsky (Berlin, Germany). AhR KO mice were obtained from Prof Gitta Stockinger (Mill Hill London, UK). MyD88 KO mice were obtained from Prof Victor Tybulewicz.

### *Mouse infection*

Anesthetised mice were infected intratracheally with the indicated doses of *A. fumigatus*, *C. albicans hgc1Δ* yeast-locked or WT (pre-formed hyphae only where indicated). Infection with *S. pneumoniae* was done intranasally by Dr Gregory T Ellis. Weight and survival of mice were monitored daily.

Where indicated 2.5 µg/g mouse neutrophil elastase inhibitor (NEi) (Sigma, GW611313A) and 0.4 µg/g mouse AREG (R&D Systems, 989-AR) were administered intraperitoneally 4 h prior to infection and subsequently every 24 h.

### *Enumerating fungal load*

To assess fungal load, mouse organs (lungs and spleens) were dissected, homogenized in sterile PBS and serial dilutions of the homogenates were spread onto sabourad dextrose agar plates. Colonies were counted after plate incubation at 37°C.

## *Microscopy*

### *Confocal microscopy of fixed cells*

$5x10^4$ human peripheral neutrophils were plated on glass coverslips 1 h prior to stimulation. After stimulation, cells were fixed with 2% paraformaldehyde for 20 min at 37°C. Immunofluorescence staining was performed as described elsewhere. In brief: cells were permeabilised using phosphate buffered saline (PBS), 0.5% Triton-X-100 for 2 min. Slides were

washed with PBS and blocked with blocking buffer (PBS, 2% BSA, 2% donkey serum) for 1 h. Slides were incubated with primary antibodies in blocking buffer for 1h, washed with PBS, incubated with secondary antibodies in blocking and DAPI in blocking buffer for 1h and washed with PBS again. Coverslips were mounted on glass microscopy slides with ProLong Gold antifade mountant (Thermo Scientific, P36934).

Primary antibodies: Anti-neutrophil elastase (NE) (Abcam, ab21595), anti-human/mouse myeloperoxidase (MPO) (R&D Systems, AF3667), anti-C. albicans (Acris, BP1006), anti-p40 (1.22; Millipore, 05-794), anti-p47 (Santa Cruz, sc-17845), anti-CD63 (RFAC4; Millipore, CBL553), DAPI (Life technologies, D1306). Secondary antibodies: donkey anti-rabbit, donkey anti-mouse coupled to AlexaFluor 488, AlexaFluor 468 or AlexaFluor 647 (all Invitrogen).

Stained cells were imaged using confocal microscopy. Images were analysed using Fiji (Schindelin, 2012).

*Confocal microscopy of tissue*

For lung histology dissected lungs of infected mice were fixed over night in 2% paraformaldehyde and embedded in wax for sectioning. Sections were treated with a standard antigen retrieval protocol and immunofluorescence staining as described elsewhere. In brief: Wax sections were baked for 60 min at 60°C. Wax was removed for 10 min in NeoClear (VWR, 1.09843.5000). Slides were rehydrated in an ethanol series (100%, 96%, 80%, 70%, 50%) for 5 min each and rinsed in aqua dist. and PBS afterwards. Antigen retrieval was done for 20 min at 95°C in 10 mM sodium carbonate buffer (0.1 M $Na_2CO_3$, 0.1 M $NaHCO_3$, 0.5 M ethylene-diamine-tetraacetic acid (EDTA), 0.05% Tween 20; pH9). Slides were cooled to room temperature and tissues were permeabilised using PBS, 0.5% Triton-X-100 for 2 min. Slides were washed with PBS and blocked with blocking buffer (PBS, 2% BSA, 2% donkey serum) for 1 h. Slides were incubated with primary antibodies in blocking

buffer for 1h, washed with PBS, incubated with secondary antibodies in blocking and DAPI in blocking buffer for 1h and washed with PBS again. Coverslips were mounted onto the slides using ProLong Gold antifade mountant (Thermo Scientific, P36934).

Cit-H3: anti-Histone H3 (citrulline R2 + R8 + R17) (Abcam, ab5103), anti-human/mouse myeloperoxidase (MPO) (R&D Systems, AF3667), DAPI (Life technologies, D1306). Secondary antibodies: donkey anti-rabbit, donkey anti-mouse coupled to AlexaFluor 488, AlexaFluor 468 or AlexaFluor 647 (all Invitrogen).

Slides were imaged using confocal microscopy. Image analysis was performed using Fiji (Schindelin, 2012).

*Confocal microscopy of live cells and M. bovis*

$6x10^5$ human peripheral neutrophils were plated with Sytox on glass bottom cell culture slides (MatTEK, CCS-8) and stimulated with a mixture of *M. bovis* BCG-dsRed single cells and aggregates. NET release was examined over a period of 4h using confocal microscopy. Time-lapse images were analysed for NET release using Fiji (Schindelin, 2012).

*Histological scores for tissue damage.*

Dissected lungs of infected mice were fixed overnight in 2% paraformaldehyde and were embedded in wax for sectioning. The sections were rehydrated as described above, were stained with hematoxilin and eosin and imaged with a VS120 virtual slide-scanning system (Olympus). 15 images of each section were randomly assigned for analysis of fibrin deposition and bleeding in the tissue according to the following scores: fibrin score, 0 (no fibrin deposition in airspace), 1 (few fibrin fibres), 2 (<50% of airspace filled with fibrin deposition) or 3 (>50% of airspace filled with fibrin deposition); and bleeding, 0 (none), 1 (mild: fewer than five red blood cells in

at least 5–10% of alveoli ), 2 (moderate: five to ten red blood cells) or 3 (severe: over ten red blood cells in >10% of alveoli).

*Immunohistochemistry*

Lung sections treated as described above were stained with antibody to tumour-necrosis factor (Abcam, ab6671) followed by a biotinylated secondary antibody (Vector Laboratories, BA-1000) amplified with Streptavidin ABC (Vector Laboratories, PK-6100), then were treated with DAB (3,3-diaminobenzidine tetrahydrochloride; Vector Laboratories, SK-4100) and imaged by light microscopy. DAB reactions were performed in parallel and at the same time for all samples.

*Quantification of NE localization*

Confocal z-series (every 0.8 μm) covering the entire neutrophil were used to quantify total NE and NE co-localizing with the nucleus using Fiji (Schindelin, 2012). For nuclear localization the NE signal was measured using a mask created with the corresponding DAPI (DNA) channel for each section. NE signals in individual sections were added to yield the total NE for each cell. 15-20 neutrophils per condition were processed.

*Live microscopy of C. albicans growth inhibition*

$2.5x10^5$ neutrophils per well were plated in HBSS++ in 8-well glass bottom dishes in the presence of DAPI and Sytox. Where indicated 50 U/ml DNase I was added. Subsequently neutrophils were stimulated with $2x10^4$ (MOI x 0.04) *C. albicans* pre-formed hyphae. Wells were imaged by inverted fluorescence microscopy over the course of 10 hours at 37°C and 5% $CO_2$.

Images were analysed using Fiji (Schindelin, 2012). Increase in hyphal length was determined using the NeuronJ plugin (Meijering, 2004).

### *Statistics*

P values were calculated by the statistic tests indicated in the figure legends of the respective experiments. P values of 0.05 or less were considered significant.

# *5 APPENDIX*

## *Abbreviations*

| | |
|---|---|
| ABAH | 4-aminobenzoic acid hydrazide |
| ACPA | anti-citrullinated peptide antibodies |
| adsA | adenosine synthase |
| AhR | aryl hydrocarbon receptor |
| AIM2 | absent in melanoma 2 |
| ANA | anti-nuclear antibodies |
| ANCA | anti-neutrophil cytoplasmic antibodies |
| AOX | alternative oxidase |
| AP-1 | activator protein 1 |
| APRIL | a proliferation-inducing ligand |
| AREG | amphiregulin |
| AZU | azurocidin |
| BAFF | B cell activating factor |
| BCG | Bacillus Calmette–Guérin (*Mycobacterim bovis* strain) |
| BCR | B cell receptor |
| BM | bone marrow |
| BUVEC | bovine umbilical vein endothelial cell |
| *C. albicans* | *Candida albicans* |
| C/EBPα | CCAAT/enhancer binding protein α |
| C5a | complement component 5a |
| CARD9 | caspase recruitment domain-containing protein 9 |
| CD | cluster of differentiation |
| CF | cystic fibrosis |
| CFU | colony-forming units |
| CG | cathepsin G |
| CGD | chronic granulomatous disease |
| citH3 | citrullinated Histone 3 |
| CLR | C-type lectin receptor |
| CPS-I | capsular polysaccharide I |
| CR3 | complement receptor 3 |

| | |
|---|---|
| CRAMP | cathelicidin-related antimicrobial peptide |
| CTLD | C-type lectin-like domain |
| CTSC | cysteine protease cathepsin C |
| dAdo | deoxyadenosine |
| DAP12 | DNAX activation protein of 12kDa |
| DAPI | 4',6-diamidino-2-phenylindole |
| DC | dendritic cell |
| DC-SIGN | dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin |
| DPI | diphenyleneiodonium |
| DPPI | dipeptidyl peptidase 1 |
| EC | epithelial cell |
| Egr2 | early growth response-2 |
| EM | erythromyeloid progenitor |
| endA | endonuclease A |
| ERK | extracellular signal-regulated kinase |
| FPR | formyl peptide receptor |
| G-CSF | granulocyte stimulating factor |
| GAS | group A *streptococcus* |
| Gfi-1 | growth factor independent-1 |
| GFTP | guanosine diphosphate-fucose transporter |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GMP | granulocyte/monocyte precursor |
| GO | glucose oxidase |
| GPCR | G-protein-coupled receptor |
| GPI | glycosylphosphatidylinositol |
| GPR43 | G-protein-coupled receptor 43 |
| HE | hematoxylin and eosin |
| HSC | hematopoietic stem cell |
| HSPC | hematopoietic stem and progenitor cell |
| ICAM-1 | intercellular adhesion molecule 1 |
| IDO | indoleamine-2,3-dioxygenase |

| | |
|---|---|
| IFN | interferon |
| IL- | interleukin |
| ILC | innate lymphoid cell |
| IRAK4 | interleukin-1 receptor-associated kinase 4 |
| IRF | interferon regulatory factor |
| ITAM | immunoreceptor tyrosine-based activation motif |
| Jak | janus kinase |
| kDa | kilo dalton |
| KO | knock-out |
| LAD | leukocyte adhesion deficiencies |
| LER | low expression region |
| LFA-1 | lymphocyte function-associated antigen 1 |
| LMP | lymphomyeloid progenitor |
| LOS | lipooligosaccharides |
| LPS | lipopolysaccharide |
| LTB4 | leukotriene B4 |
| lukGH | leukotoxin GH |
| Mac-1 | macrophage-1 antigen |
| MAPK | mitogen-activated protein kinase |
| MDA5 | melanoma differentiation antigen 5 |
| MDP | muramyl dipeptide |
| MDSC | myeloid-derived suppressor cell |
| MEK | mitogen-activated protein kinase kinase |
| MHC | major histocompatibility complex |
| MMP-8 | metalloprotease-8 |
| MNase | micrococcal nuclease |
| MOI | multiplicity of infection |
| MPO | myeloperoxidase |
| MPP | multipotent precursor |
| MR | mannose receptor |
| MRP | myeloid related protein |
| MSU | monosodium urate |

| | |
|---|---|
| MyD88 | myeloid differentiation primary response gene 88 |
| MZ | marginal zone |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NE | neutrophil elastase |
| NEi | neutrophil elastase inhibitor |
| NET | neutrophil extracellular trap |
| NF-κB | nuclear factor-κB |
| NFAT | nuclear factor of activated T-cells |
| NK cell | natural killer cell |
| NLR | nucleotide-binding oligomerisation domain (NOD)-like receptor |
| NLRP3 | NOD-, LRR- and pyrin domain-containing 3 |
| NS | not significant |
| NTHI | nontypeable *Haemophilus influenzae* |
| OPC | oropharyngal candidiasis |
| PAD4 | peptidylarginine deiminase 4 |
| PAF | platelet activating factor |
| PAMP | pathogen associated molecular pattern |
| PD-L1 | programmed death-ligand 1 |
| pDCs | plasmacytoid dendritic cells |
| PI3K | phosphoinositide 3-kinase |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PLC | phospholipase C |
| PLS | Papillon-Lefèvre syndrome |
| PMA | phorbol myristate acetate |
| PMN | polymorphnuclear cell |
| PPARγ | peroxisome proliferator activated receptor-γ |
| PR3 | proteinase 3 |
| PRR | pattern recognition receptor |
| PSGL-1 | P-selectin glycoprotein ligand-1 |
| PSMγ | phenol-soluble modulin δ-toxin |

| | |
|---|---|
| RA | rheumatoid arthritis |
| RIG-I | retinoic acid-inducible gene 1 |
| RIP2 | receptor-interacting serine/threonine protein kinase 2 |
| ROS | reactive oxygen species |
| RSV | respiratory syncytial virus |
| SC | stem cell |
| SCAMP | secretory carrier membrane protein |
| SCN | severe congenital neutropenia |
| SDF1 | stromal derived factor 1 |
| SLE | systemic lupus erythematosus |
| SNARE | soluble N-methylmaleimide-sensitive factor attachment protein (SNAP)–receptor |
| SOD | superoxide dismutase |
| SsnA | secreted nuclease A |
| STAT1 | signal transducer and activator of transcription-1 |
| Syk | spleen tyrosine kinase |
| TCR | T cell receptor |
| TEM | transendothelial migration |
| TLR | Toll-like receptor |
| TNFα | tumour necrosis factor α |
| TRIF | TIR-domain-containing adapter-inducing interferon-β |
| Unst | unstimulated |
| Untr | untreated |
| UPR | unfolded protein response |
| VAMP-2 | vesicle-associated membrane protein 2 |
| VLA-4 | very late antigen-4 |
| VVO | vesiculovacuolar organelle |
| vWF | van Willebrand factor |
| WHIM | warts, hypogammaglobulinemia, infections, myelokathexis |
| WT | wild-type |
| XTT | 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide |

## *List of figures*

**Introduction**



**Results**

# *6 REFERENCES*

**Abi Abdallah, D.S., Lin, C., Ball, C.J., King, M.R., Duhamel, G.E.*, et al.*** (2012). Toxoplasma gondii triggers release of human and mouse neutrophil extracellular traps. Infect Immun *80*, 768-77.

**Achouiti, A., Vogl, T., Urban, C.F., Rohm, M., Hommes, T.J.*, et al.*** (2012). Myeloid-related protein-14 contributes to protective immunity in gram-negative pneumonia derived sepsis. PLoS Pathog *8*, e1002987.

**Acosta-Rodriguez, E.V., Rivino, L., Geginat, J., Jarrossay, D., Gattorno, M.*, et al.*** (2007). Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol *8*, 639-46.

**Aga, E., Katschinski, D.M., van Zandbergen, G., Laufs, H., Hansen, B.*, et al.*** (2002). Inhibition of the Spontaneous Apoptosis of Neutrophil Granulocytes by the Intracellular Parasite Leishmania major. J Immunol *169*, 898-905.

**Akong-Moore, K., Chow, O.A., von Kockritz-Blickwede, M., and Nizet, V.** (2012). Influences of chloride and hypochlorite on neutrophil extracellular trap formation. PLoS One *7*, e42984.

**Al-Homood, I.A.** (2012). Thrombosis in systemic lupus erythematosus: a review article. ISRN Rheumatol *2012*, 428269.

**Alalwani, S.M., Sierigk, J., Herr, C., Pinkenburg, O., Gallo, R.*, et al.*** (2010). The antimicrobial peptide LL-37 modulates the inflammatory and host defense response of human neutrophils. Eur J Immunol *40*, 1118-26.

**Aleman, O.R., Mora, N., Cortes-Vieyra, R., Uribe-Querol, E., and Rosales, C.** (2016). Differential Use of Human Neutrophil Fc Receptors for Inducing Neutrophil Extracellular Trap Formation. J Immunol Res *2016*, 2908034.

**Allen, I.C., Scull, M.A., Moore, C.B., Holl, E.K., McElvania-TeKippe, E.*, et al.*** (2009). The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. Immunity *30*, 556-65.

**Allen, L.A., DeLeo, F.R., Gallois, A., Toyoshima, S., Suzuki, K.*, et al.*** (1999). Transient association of the nicotinamide adenine dinucleotide phosphate oxidase subunits p47phox and p67phox with phagosomes in neutrophils from patients with X-linked chronic granulomatous disease. Blood *93*, 3521-30.

**Allingham, M.J., van Buul, J.D., and Burridge, K.** (2007). ICAM-1-mediated, Src- and Pyk2-dependent vascular endothelial cadherin tyrosine phosphorylation is required for leukocyte transendothelial migration. J Immunol *179*, 4053-64.

**Alon, R., and Feigelson, S.W.** (2009). Chemokine signaling to lymphocyte integrins under shear flow. Microcirculation *16*, 3-16.

**Ammollo, C.T., Semeraro, F., Xu, J., Esmon, N.L., and Esmon, C.T.** (2011). Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein C activation. J Thromb Haemost *9*, 1795-803.

**Antachopoulos, C.** (2010). Invasive fungal infections in congenital immunodeficiencies. Clin Microbiol Infect *16*, 1335-42.

**Arinobu, Y., Mizuno, S., Chong, Y., Shigematsu, H., Iino, T.*, et al.*** (2007). Reciprocal activation of GATA-1 and PU.1 marks initial specification of hematopoietic stem cells into myeloerythroid and myelolymphoid lineages. Cell Stem Cell *1*, 416-27.

**Aujla, S.J., Dubin, P.J., and Kolls, J.K.** (2007). Th17 cells and mucosal host defense. Semin Immunol *19*, 377-82.

**Aulik, N.A., Hellenbrand, K.M., Klos, H., and Czuprynski, C.J.** (2010). Mannheimia haemolytica and its leukotoxin cause neutrophil extracellular trap formation by bovine neutrophils. Infect Immun *78*, 4454-66.

**Ayres-Sander, C.E., Lauridsen, H., Maier, C.L., Sava, P., Pober, J.S.*, et al.*** (2013). Transendothelial migration enables subsequent transmigration of neutrophils through underlying pericytes. PLoS One *8*, e60025.

**Babior, B.M.** (2004). NADPH oxidase. Curr Opin Immunol *16*, 42-7.

**Babior, B.M., Lambeth, J.D., and Nauseef, W.** (2002). The neutrophil NADPH oxidase. Arch Biochem Biophys *397*, 342-4.

**Bachiega, T.F., Dias-Melicio, L.A., Fernandes, R.K., de Almeida Balderramas, H., Rodrigues, D.R.*, et al.*** (2016). Participation of dectin-1 receptor on NETs release against Paracoccidioides brasiliensis: Role on extracellular killing. Immunobiology *221*, 228-35.

**Bainton, D.F., Ullyot, J.L., and Farquhar, M.G.** (1971). The development of neutrophilic polymorphonuclear leukocytes in human bone marrow: origin and content of azurophil and specific granules. J Exp Med *134*, 907-34.

**Baker, V.S., Imade, G.E., Molta, N.B., Tawde, P., Pam, S.D.*, et al.*** (2008). Cytokine-associated neutrophil extracellular traps and antinuclear antibodies in Plasmodium falciparum infected children under six years of age. Malar J *7*, 41.

**Balmer, M.L., Schurch, C.M., Saito, Y., Geuking, M.B., Li, H.*, et al.*** (2014). Microbiota-derived compounds drive steady-state granulopoiesis via MyD88/TICAM signaling. J Immunol *193*, 5273-83.

**Bar, E., Whitney, P.G., Moor, K., Reis e Sousa, C., and LeibundGut-Landmann, S.** (2014). IL-17 regulates systemic fungal immunity by controlling the functional competence of NK cells. Immunity *40*, 117-27.

**Bardoel, B.W., Kenny, E.F., Sollberger, G., and Zychlinsky, A.** (2014). The balancing act of neutrophils. Cell Host Microbe *15*, 526-36.

**Basu, S., Hodgson, G., Zhang, H.-H., Katz, M., Quilici, C.*, et al.*** (2000). "Emergency" granulopoiesis in G-CSF–deficient mice in response to Candida albicans infection. Blood *95*, 3725-33.

**Bates, S., Hughes, H.B., Munro, C.A., Thomas, W.P., MacCallum, D.M.*, et al.*** (2006). Outer chain N-glycans are required for cell wall integrity and virulence of Candida albicans. J Biol Chem *281*, 90-8.

**Bates, S., MacCallum, D.M., Bertram, G., Munro, C.A., Hughes, H.B.*, et al.*** (2005). Candida albicans Pmr1p, a secretory pathway P-type Ca2+/Mn2+-ATPase, is required for glycosylation and virulence. J Biol Chem *280*, 23408-15.

**Beausejour, A., Grenier, D., Goulet, J.P., and Deslauriers, N.** (1998). Proteolytic activation of the interleukin-1beta precursor by Candida albicans. Infect Immun *66*, 676-81.

**Beauvillain, C., Cunin, P., Doni, A., Scotet, M., Jaillon, S.*, et al.*** (2011). CCR7 is involved in the migration of neutrophils to lymph nodes. Blood *117*, 1196-204.

**Behrendt, J.H., Ruiz, A., Zahner, H., Taubert, A., and Hermosilla, C.** (2010). Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite Eimeria bovis. Vet Immunol Immunopathol *133*, 1-8.

**Beiter, K., Wartha, F., Albiger, B., Normark, S., Zychlinsky, A.*, et al.*** (2006). An endonuclease allows Streptococcus pneumoniae to escape from neutrophil extracellular traps. Curr Biol *16*, 401-7.

**Bellocchio, S., Montagnoli, C., Bozza, S., Gaziano, R., Rossi, G.*, et al.*** (2004). The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. J Immunol *172*, 3059-69.

**Benghezal, M., Adam, E., Lucas, A., Burn, C., Orchard, M.G.*, et al.*** (2007). Inhibitors of bacterial virulence identified in a surrogate host model. Cell Microbiol *9*, 1336-42.

**Berends, E.T., Horswill, A.R., Haste, N.M., Monestier, M., Nizet, V.*, et al.*** (2010). Nuclease expression by Staphylococcus aureus facilitates escape from neutrophil extracellular traps. J Innate Immun *2*, 576-86.

**Berger-Achituv, S., Brinkmann, V., Abed, U.A., Kuhn, L.I., Ben-Ezra, J.*, et al.*** (2013). A proposed role for neutrophil extracellular traps in cancer immunoediting. Front Immunol *4*, 48.

**Bernut, A., Herrmann, J.L., Kissa, K., Dubremetz, J.F., Gaillard, J.L.*, et al.*** (2014). Mycobacterium abscessus cording prevents phagocytosis and promotes abscess formation. Proc Natl Acad Sci U S A *111*, E943-52.

**Berthier, S., Paclet, M.H., Lerouge, S., Roux, F., Vergnaud, S.*, et al.*** (2003). Changing the conformation state of cytochrome b558 initiates NADPH oxidase activation: MRP8/MRP14 regulation. J Biol Chem *278*, 25499-508.

**Bianchi, M., Hakkim, A., Brinkmann, V., Siler, U., Seger, R.A.*, et al.*** (2009). Restoration of NET formation by gene therapy in CGD controls aspergillosis. Blood *114*, 2619-22.

**Bianchi, M., Niemiec, M.J., Siler, U., Urban, C.F., and Reichenbach, J.** (2011). Restoration of anti-Aspergillus defense by neutrophil extracellular traps in human chronic granulomatous disease after gene therapy is calprotectin-dependent. J Allergy Clin Immunol *127*, 1243-52 e7.

**Biondo, C., Midiri, A., Messina, L., Tomasello, F., Garufi, G.*, et al.*** (2005). MyD88 and TLR2, but not TLR4, are required for host defense against Cryptococcus neoformans. Eur J Immunol *35*, 870-8.

**Bishop, A., Lane, R., Beniston, R., Chapa-y-Lazo, B., Smythe, C.*, et al.*** (2010). Hyphal growth in Candida albicans requires the phosphorylation of Sec2 by the Cdc28-Ccn1/Hgc1 kinase. EMBO J *29*, 2930-42.

**Bjerregaard, M.D., Jurlander, J., Klausen, P., Borregaard, N., and Cowland, J.B.** (2003). The in vivo profile of transcription factors during neutrophil differentiation in human bone marrow. Blood *101*, 4322-32.

**Blander, J.M., and Medzhitov, R.** (2006). On regulation of phagosome maturation and antigen presentation. Nat Immunol *7*, 1029-35.

**Bochud, P.Y., Chien, J.W., Marr, K.A., Leisenring, W.M., Upton, A.*, et al.*** (2008). Toll-like receptor 4 polymorphisms and aspergillosis in stem-cell transplantation. N Engl J Med *359*, 1766-77.

**Bockmuhl, D.P., and Ernst, J.F.** (2001). A potential phosphorylation site for an A-type kinase in the Efg1 regulator protein contributes to hyphal morphogenesis of Candida albicans. Genetics *157*, 1523-30.

**Bonifazi, P., Zelante, T., D'Angelo, C., De Luca, A., Moretti, S.*, et al.*** (2009). Balancing inflammation and tolerance in vivo through dendritic cells by the commensal Candida albicans. Mucosal Immunol *2*, 362-74.

**Bonne-Annee, S., Kerepesi, L.A., Hess, J.A., Wesolowski, J., Paumet, F.*, et al.*** (2014). Extracellular traps are associated with human and mouse neutrophil and macrophage mediated killing of larval Strongyloides stercoralis. Microbes Infect *16*, 502-11.

**Borissoff, J.I., Joosen, I.A., Versteylen, M.O., Brill, A., Fuchs, T.A.*, et al.*** (2013). Elevated levels of circulating DNA and chromatin are independently associated with severe coronary atherosclerosis and a prothrombotic state. Arterioscler Thromb Vasc Biol *33*, 2032-40.

**Borregaard, N., and Cowland, J.B.** (1997). Granules of the human neutrophilic polymorphonuclear leukocyte. Blood *89*, 3503-21.

**Borregaard, N., Sorensen, O.E., and Theilgaard-Monch, K.** (2007). Neutrophil granules: a library of innate immunity proteins. Trends Immunol *28*, 340-5.

**Borregaard, N., and Tauber, A.I.** (1984). Subcellular localization of the human neutrophil NADPH oxidase. b-Cytochrome and associated flavoprotein. J Biol Chem *259*, 47-52.

**Botto, M., Dell'Agnola, C., Bygrave, A.E., Thompson, E.M., Cook, H.T.*, et al.*** (1998). Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat Genet *19*, 56-9.

**Bourgeois, C., and Kuchler, K.** (2012). Fungal pathogens-a sweet and sour treat for toll-like receptors. Front Cell Infect Microbiol *2*, 142.

**Bourgeois, C., Majer, O., Frohner, I.E., Lesiak-Markowicz, I., Hildering, K.S.*, et al.*** (2011). Conventional dendritic cells mount a type I IFN response against Candida spp. requiring novel phagosomal TLR7-mediated IFN-beta signaling. J Immunol *186*, 3104-12.

**Bourgeois, C., Majer, O., Frohner, I.E., Tierney, L., and Kuchler, K.** (2010). Fungal attacks on mammalian hosts: pathogen elimination requires sensing and tasting. Curr Opin Microbiol *13*, 401-8.

**Brand, A., MacCallum, D.M., Brown, A.J., Gow, N.A., and Odds, F.C.** (2004). Ectopic expression of URA3 can influence the virulence phenotypes and proteome of Candida albicans but can be overcome by targeted reintegration of URA3 at the RPS10 locus. Eukaryot Cell *3*, 900-9.

**Brandau, S., Dumitru, C.A., and Lang, S.** (2013). Protumor and antitumor functions of neutrophil granulocytes. Semin Immunopathol *35*, 163-76.

**Branzk, N., Lubojemska, A., Hardison, S.E., Wang, Q., Gutierrez, M.G.*, et al.*** (2014). Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. Nat Immunol *15*, 1017-25.

**Branzk, N., and Papayannopoulos, V.** (2013). Molecular mechanisms regulating NETosis in infection and disease. Semin Immunopathol *35*, 513-30.

**Bretz, C., Gersuk, G., Knoblaugh, S., Chaudhary, N., Randolph-Habecker, J.*, et al.*** (2008). MyD88 signaling contributes to early pulmonary responses to Aspergillus fumigatus. Infect Immun *76*, 952-8.

**Brill, A., Fuchs, T.A., Savchenko, A.S., Thomas, G.M., Martinod, K.*, et al.*** (2012). Neutrophil extracellular traps promote deep vein thrombosis in mice. Journal of Thrombosis and Haemostasis *10*, 136-44.

**Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y.*, et al.*** (2004). Neutrophil extracellular traps kill bacteria. Science *303*, 1532-5.

**Broermann, A., Winderlich, M., Block, H., Frye, M., Rossaint, J.*, et al.*** (2011). Dissociation of VE-PTP from VE-cadherin is required for leukocyte extravasation and for VEGF-induced vascular permeability in vivo. J Exp Med *208*, 2393-401.

**Brown, A.J., Odds, F.C., and Gow, N.A.** (2007). Infection-related gene expression in Candida albicans. Curr Opin Microbiol *10*, 307-13.

**Brown, G.D.** (2011). Innate antifungal immunity: the key role of phagocytes. Annu Rev Immunol *29*, 1-21.

**Brown, G.D., Denning, D.W., Gow, N.A., Levitz, S.M., Netea, M.G.*, et al.*** (2012a). Hidden killers: human fungal infections. Sci Transl Med *4*, 165rv13.

**Brown, G.D., Denning, D.W., and Levitz, S.M.** (2012b). Tackling human fungal infections. Science *336*, 647.

**Brumell, J.H., Volchuk, A., Sengelov, H., Borregaard, N., Cieutat, A.M.*, et al.*** (1995). Subcellular distribution of docking/fusion proteins in neutrophils, secretory cells with multiple exocytic compartments. J Immunol *155*, 5750-9.

**Brunke, S., and Hube, B.** (2013). Two unlike cousins: Candida albicans and C. glabrata infection strategies. Cell Microbiol *15*, 701-8.

**Bruns, S., Kniemeyer, O., Hasenberg, M., Aimanianda, V., Nietzsche, S.*, et al.*** (2010). Production of extracellular traps against Aspergillus fumigatus in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. PLoS Pathog *6*, e1000873.

**Buchanan, J.T., Simpson, A.J., Aziz, R.K., Liu, G.Y., Kristian, S.A.*, et al.*** (2006). DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. Curr Biol *16*, 396-400.

**Bugl, S., Wirths, S., Muller, M.R., Radsak, M.P., and Kopp, H.G.** (2012). Current insights into neutrophil homeostasis. Ann N Y Acad Sci *1266*, 171-8.

**Bugl, S., Wirths, S., Radsak, M.P., Schild, H., Stein, P.*, et al.*** (2013). Steady-state neutrophil homeostasis is dependent on TLR4/TRIF signaling. Blood *121*, 723-33.

**Byrd, A.S., O'Brien, X.M., Johnson, C.M., Lavigne, L.M., and Reichner, J.S.** (2013). An extracellular matrix-based mechanism of rapid neutrophil extracellular trap formation in response to Candida albicans. J Immunol *190*, 4136-48.

**Byrd, A.S., O'Brien, X.M., Laforce-Nesbitt, S.S., Parisi, V.E., Hirakawa, M.P.*, et al.*** (2015). NETosis in Neonates: Evidence of a Reactive Oxygen Species-Independent Pathway in Response to Fungal Challenge. J Infect Dis.

**Cantin, A.M.** (1998). DNase I acutely increases cystic fibrosis sputum elastase activity and its potential to induce lung hemorrhage in mice. Am J Respir Crit Care Med *157*, 464-9.

**Carlin, A.F., Uchiyama, S., Chang, Y.C., Lewis, A.L., Nizet, V.*, et al.*** (2009). Molecular mimicry of host sialylated glycans allows a bacterial pathogen to engage neutrophil Siglec-9 and dampen the innate immune response. Blood *113*, 3333-6.

**Carman, C.V., Sage, P.T., Sciuto, T.E., de la Fuente, M.A., Geha, R.S.*, et al.*** (2007). Transcellular diapedesis is initiated by invasive podosomes. Immunity *26*, 784-97.

**Carvalho, A., De Luca, A., Bozza, S., Cunha, C., D'Angelo, C.*, et al.*** (2012). TLR3 essentially promotes protective class I-restricted memory CD8(+) T-cell responses to Aspergillus fumigatus in hematopoietic transplanted patients. Blood *119*, 967-77.

**Carvalho, A., Pasqualotto, A.C., Pitzurra, L., Romani, L., Denning, D.W.*, et al.*** (2008). Polymorphisms in toll-like receptor genes and susceptibility to pulmonary aspergillosis. J Infect Dis *197*, 618-21.

**Casanova-Acebes, M., Pitaval, C., Weiss, L.A., Nombela-Arrieta, C., Chevre, R.*, et al.*** (2013). Rhythmic modulation of the hematopoietic niche through neutrophil clearance. Cell *153*, 1025-35.

**Casutt-Meyer, S., Renzi, F., Schmaler, M., Jann, N.J., Amstutz, M.*, et al.*** (2010). Oligomeric coiled-coil adhesin YadA is a double-edged sword. PLoS One *5*, e15159.

**Cerutti, A., Puga, I., and Magri, G.** (2013). The B cell helper side of neutrophils. J Leukoc Biol *94*, 677-82.

**Chang, X., Yamada, R., Suzuki, A., Sawada, T., Yoshino, S.*, et al.*** (2005). Localization of peptidylarginine deiminase 4 (PADI4) and citrullinated protein in synovial tissue of rheumatoid arthritis. Rheumatology (Oxford) *44*, 40-50.

**Charmoy, M., Megnekou, R., Allenbach, C., Zweifel, C., Perez, C.*, et al.*** (2007). Leishmania major induces distinct neutrophil phenotypes in mice that are resistant or susceptible to infection. J Leukoc Biol *82*, 288-99.

**Chen, S., and Springer, T.A.** (1999). An automatic braking system that stabilizes leukocyte rolling by an increase in selectin bond number with shear. J Cell Biol *144*, 185-200.

**Cheng, J.J., Yang, C.J., Cheng, C.H., Wang, Y.T., Huang, N.K.*, et al.*** (2005). Characterization and functional study of Antrodia camphorata lipopolysaccharide. J Agric Food Chem *53*, 469-74.

**Cheng, S.C., Quintin, J., Cramer, R.A., Shepardson, K.M., Saeed, S.*, et al.*** (2014). mTOR- and HIF-1 -mediated aerobic glycolysis as metabolic basis for trained immunity. Science *345*, 1250684-84.

**Cheng, S.C., van de Veerdonk, F., Smeekens, S., Joosten, L.A., van der Meer, J.W.*, et al.*** (2010). Candida albicans dampens host defense by downregulating IL-17 production. J Immunol *185*, 2450-7.

**Cheng, S.C., van de Veerdonk, F.L., Lenardon, M., Stoffels, M., Plantinga, T.*, et al.*** (2011). The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of Candida albicans. J Leukoc Biol *90*, 357-66.

**Chi, H., and Sun, L.** (2016). Neutrophils of Scophthalmus maximus produce extracellular traps that capture bacteria and inhibit bacterial infection. Dev Comp Immunol *56*, 7-12.

**Christopher, M.J., Liu, F., Hilton, M.J., Long, F., and Link, D.C.** (2009). Suppression of CXCL12 production by bone marrow osteoblasts is a common and critical pathway for cytokine-induced mobilization. Blood *114*, 1331-9.

**Chumakov, A.M., Silla, A., Williamson, E.A., and Koeffler, H.P.** (2007). Modulation of DNA binding properties of CCAAT/enhancer binding protein epsilon by heterodimer formation and interactions with NFkappaB pathway. Blood *109*, 4209-19.

**Clark, H.L., Jhingran, A., Sun, Y., Vareechon, C., de Jesus Carrion, S.*, et al.*** (2015). Zinc and Manganese Chelation by Neutrophil S100A8/A9 (Calprotectin) Limits Extracellular Aspergillus fumigatus Hyphal Growth and Corneal Infection. J Immunol.

**Clarke, T.B., Davis, K.M., Lysenko, E.S., Zhou, A.Y., Yu, Y.*, et al.*** (2010). Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat Med *16*, 228-31.

**Claudia, M., Bacci, A., Silvia, B., Gaziano, R., Spreca, A.*, et al.*** (2002). The interaction of fungi with dendritic cells: implications for Th immunity and vaccination. Curr Mol Med *2*, 507-24.

**Cogen, A.L., Yamasaki, K., Muto, J., Sanchez, K.M., Crotty Alexander, L.*, et al.*** (2010). Staphylococcus epidermidis antimicrobial delta-toxin (phenol-soluble modulin-gamma) cooperates with host antimicrobial peptides to kill group A Streptococcus. PLoS One *5*, e8557.

**Cohen, N.R., Tatituri, R.V., Rivera, A., Watts, G.F., Kim, E.Y.*, et al.*** (2011). Innate recognition of cell wall beta-glucans drives invariant natural killer T cell responses against fungi. Cell Host Microbe *10*, 437-50.

**Condliffe, A.M., Kitchen, E., and Chilvers, E.R.** (1998). Neutrophil priming: pathophysiological consequences and underlying mechanisms. Clin Sci (Lond) *94*, 461-71.

**Conti, H.R., Shen, F., Nayyar, N., Stocum, E., Sun, J.N.*, et al.*** (2009). Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. J Exp Med *206*, 299-311.

**Cools-Lartigue, J., Spicer, J., McDonald, B., Gowing, S., Chow, S.*, et al.*** (2013). Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. J Clin Invest.

**Cools-Lartigue, J., Spicer, J., Najmeh, S., and Ferri, L.** (2014). Neutrophil extracellular traps in cancer progression. Cell Mol Life Sci *71*, 4179-94.

**Craven, R.R., Gao, X., Allen, I.C., Gris, D., Bubeck Wardenburg, J.*, et al.*** (2009). Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. PLoS One *4*, e7446.

**Cua, D.J., and Tato, C.M.** (2010). Innate IL-17-producing cells: the sentinels of the immune system. Nat Rev Immunol *10*, 479-89.

**Cunha, C., Di Ianni, M., Bozza, S., Giovannini, G., Zagarella, S.*, et al.*** (2010). Dectin-1 Y238X polymorphism associates with susceptibility to invasive aspergillosis in hematopoietic transplantation through impairment of both recipient- and donor-dependent mechanisms of antifungal immunity. Blood *116*, 5394-402.

**Cutler, J.E.** (2001). N-glycosylation of yeast, with emphasis on Candida albicans. Med Mycol *39 Suppl 1*, 75-86.

**d'Ostiani, C.F., Del Sero, G., Bacci, A., Montagnoli, C., Spreca, A.*, et al.*** (2000). Dendritic cells discriminate between yeasts and hyphae of the fungus Candida albicans. Implications for initiation of T helper cell immunity in vitro and in vivo. J Exp Med *191*, 1661-74.

**Dahl, R., Walsh, J.C., Lancki, D., Laslo, P., Iyer, S.R.*, et al.*** (2003). Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. Nat Immunol *4*, 1029-36.

**Dale, D.C., Person, R.E., Bolyard, A.A., Aprikyan, A.G., Bos, C.*, et al.*** (2000). Mutations in the gene encoding neutrophil elastase in congenital and cyclic neutropenia. Blood *96*, 2317-22.

**de Buhr, N., Neumann, A., Jerjomiceva, N., von Kockritz-Blickwede, M., and Baums, C.G.** (2014). Streptococcus suis DNase SsnA contributes to degradation of neutrophil extracellular traps (NETs) and evasion of NET-mediated antimicrobial activity. Microbiology *160*, 385-95.

**de Buhr, N., Stehr, M., Neumann, A., Naim, H.Y., Valentin-Weigand, P.*, et al.*** (2015). Identification of a novel DNase of Streptococcus suis (EndAsuis) important for neutrophil extracellular trap degradation during exponential growth. Microbiology *161*, 838-50.

**de Haar, S.F., Hiemstra, P.S., van Steenbergen, M.T., Everts, V., and Beertsen, W.** (2006). Role of polymorphonuclear leukocyte-derived serine proteinases in defense against Actinobacillus actinomycetemcomitans. Infect Immun *74*, 5284-91.

**Decoursey, T.E., and Ligeti, E.** (2005). Regulation and termination of NADPH oxidase activity. Cell Mol Life Sci *62*, 2173-93.

**del Fresno, C., Soulat, D., Roth, S., Blazek, K., Udalova, I.*, et al.*** (2013). Interferon-beta production via Dectin-1-Syk-IRF5 signaling in dendritic cells is crucial for immunity to C. albicans. Immunity *38*, 1176-86.

**Della Coletta, A.M., Bachiega, T.F., de Quaglia e Silva, J.C., Soares, A.M., De Faveri, J.*, et al.*** (2015). Neutrophil Extracellular Traps Identification in Tegumentary Lesions of Patients with Paracoccidioidomycosis and Different Patterns of NETs Generation In Vitro. PLoS Negl Trop Dis *9*, e0004037.

**Denny, M.F., Yalavarthi, S., Zhao, W., Thacker, S.G., Anderson, M.*, et al.*** (2010). A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. J Immunol *184*, 3284-97.

**Deshmukh, H.S., Liu, Y., Menkiti, O.R., Mei, J., Dai, N.*, et al.*** (2014). The microbiota regulates neutrophil homeostasis and host resistance to Escherichia coli K1 sepsis in neonatal mice. Nat Med *20*, 524-30.

**Deveau, A., Piispanen, A.E., Jackson, A.A., and Hogan, D.A.** (2010). Farnesol induces hydrogen peroxide resistance in Candida albicans yeast by inhibiting the Ras-cyclic AMP signaling pathway. Eukaryot Cell *9*, 569-77.

**Diamond, R.D., and Clark, R.A.** (1982). Damage to Aspergillus fumigatus and Rhizopus oryzae hyphae by oxidative and nonoxidative microbicidal products of human neutrophils in vitro. Infect Immun *38*, 487-95.

**Ding, L., and Morrison, S.J.** (2013). Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. Nature *495*, 231-5.

**Ding, L., Saunders, T.L., Enikolopov, G., and Morrison, S.J.** (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. Nature *481*, 457-62.

**Dixit, N., and Simon, S.I.** (2012). Chemokines, selectins and intracellular calcium flux: temporal and spatial cues for leukocyte arrest. Front Immunol *3*, 188.

**Dongari-Bagtzoglou, A., and Fidel, P.L., Jr.** (2005). The host cytokine responses and protective immunity in oropharyngeal candidiasis. J Dent Res *84*, 966-77.

**Doring, Y., Drechsler, M., Wantha, S., Kemmerich, K., Lievens, D.*, et al.*** (2012a). Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice. Circ Res *110*, 1052-6.

**Doring, Y., Manthey, H.D., Drechsler, M., Lievens, D., Megens, R.T.*, et al.*** (2012b). Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. Circulation *125*, 1673-83.

**Dostert, C., Guarda, G., Romero, J.F., Menu, P., Gross, O.*, et al.*** (2009). Malarial hemozoin is a Nalp3 inflammasome activating danger signal. PLoS One *4*, e6510.

**Dostert, C., Petrilli, V., Van Bruggen, R., Steele, C., Mossman, B.T.*, et al.*** (2008). Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science *320*, 674-7.

**Drago, L., Bortolin, M., Vassena, C., Taschieri, S., and Del Fabbro, M.** (2013). Antimicrobial activity of pure platelet-rich plasma against microorganisms isolated from oral cavity. BMC Microbiol *13*, 47.

**Drewniak, A., Gazendam, R.P., Tool, A.T., van Houdt, M., Jansen, M.H.*, et al.*** (2013). Invasive fungal infection and impaired neutrophil killing in human CARD9 deficiency. Blood *121*, 2385-92.

**Drummond, R.A., and Brown, G.D.** (2011). The role of Dectin-1 in the host defence against fungal infections. Curr Opin Microbiol *14*, 392-9.

**Drummond, R.A., Saijo, S., Iwakura, Y., and Brown, G.D.** (2011). The role of Syk/CARD9 coupled C-type lectins in antifungal immunity. Eur J Immunol *41*, 276-81.

**Duffy, D., Perrin, H., Abadie, V., Benhabiles, N., Boissonnas, A.*, et al.*** (2012). Neutrophils transport antigen from the dermis to the bone marrow, initiating a source of memory CD8+ T cells. Immunity *37*, 917-29.

**Eash, K.J., Means, J.M., White, D.W., and Link, D.C.** (2009). CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. Blood *113*, 4711-9.

**Edelstein, A.D., Tsuchida, M.A., Amodaj, N., Pinkard, H., Vale, R.D.*, et al.*** (2014). Advanced methods of microscope control using muManager software. J Biol Methods *1*.

**Ekman, A.K., and Cardell, L.O.** (2010). The expression and function of Nod-like receptors in neutrophils. Immunology *130*, 55-63.

**Ellis, G.T., Davidson, S., Crotta, S., Branzk, N., Papayannopoulos, V.*, et al.*** (2015). TRAIL+ monocytes and monocyte-related cells cause lung damage and thereby increase susceptibility to influenza-Streptococcus pneumoniae coinfection. EMBO Rep *16*, 1203-18.

**Ermert, D., Urban, C.F., Laube, B., Goosmann, C., Zychlinsky, A.*, et al.*** (2009). Mouse Neutrophil Extracellular Traps in Microbial Infections. J Innate Immun *1*, 181-93.

**Ernst, J.F., and Prill, S.K.** (2001). O-glycosylation. Med Mycol *39 Suppl 1*, 67-74.

**Eyerich, S., Wagener, J., Wenzel, V., Scarponi, C., Pennino, D.*, et al.*** (2011). IL-22 and TNF-alpha represent a key cytokine combination for epidermal integrity during infection with Candida albicans. Eur J Immunol *41*, 1894-901.

**Farrera, C., and Fadeel, B.** (2013). Macrophage clearance of neutrophil extracellular traps is a silent process. J Immunol *191*, 2647-56.

**Faure, M.C., Sulpice, J.C., Delattre, M., Lavielle, M., Prigent, M.*, et al.*** (2013). The recruitment of p47(phox) and Rac2G12V at the phagosome is transient and phosphatidylserine dependent. Biol Cell *105*, 501-18.

**Feriotti, C., Loures, F.V., Frank de Araujo, E., da Costa, T.A., and Calich, V.L.** (2013). Mannosyl-recognizing receptors induce an M1-like phenotype in macrophages of susceptible mice but an M2-like phenotype in mice resistant to a fungal infection. PLoS One *8*, e54845.

**Ferreira, M.C., de Oliveira, R.T., da Silva, R.M., Blotta, M.H., and Mamoni, R.L.** (2010). Involvement of regulatory T cells in the immunosuppression characteristic of patients with paracoccidioidomycosis. Infect Immun *78*, 4392-401.

**Ferwerda, B., Ferwerda, G., Plantinga, T.S., Willment, J.A., van Spriel, A.B.*, et al.*** (2009). Human dectin-1 deficiency and mucocutaneous fungal infections. N Engl J Med *361*, 1760-7.

**Flajnik, M.F., and Kasahara, M.** (2010). Origin and evolution of the adaptive immune system: genetic events and selective pressures. Nat Rev Genet *11*, 47-59.

**Fonzi, W.A., and Irwin, M.Y.** (1993). Isogenic strain construction and gene mapping in Candida albicans. Genetics *134*, 717-28.

**Foxman, E.F., Campbell, J.J., and Butcher, E.C.** (1997). Multistep navigation and the combinatorial control of leukocyte chemotaxis. J Cell Biol *139*, 1349-60.

**Francis, R.J., Butler, R.E., and Stewart, G.R.** (2014). Mycobacterium tuberculosis ESAT-6 is a leukocidin causing Ca2+ influx, necrosis and neutrophil extracellular trap formation. Cell Death Dis *5*, e1474.

**Fritz, J.H., Ferrero, R.L., Philpott, D.J., and Girardin, S.E.** (2006). Nod-like proteins in immunity, inflammation and disease. Nat Immunol *7*, 1250-7.

**Fuchs, T.A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I.*, et al.*** (2007). Novel cell death program leads to neutrophil extracellular traps. J Cell Biol *176*, 231-41.

**Fuller, G.L., Williams, J.A., Tomlinson, M.G., Eble, J.A., Hanna, S.L.*, et al.*** (2007). The C-type lectin receptors CLEC-2 and Dectin-1, but not DC-SIGN, signal via a novel YXXL-dependent signaling cascade. J Biol Chem *282*, 12397-409.

**Funchal, G.A., Jaeger, N., Czepielewski, R.S., Machado, M.S., Muraro, S.P.*, et al.*** (2015). Respiratory syncytial virus fusion protein promotes TLR-4-dependent neutrophil extracellular trap formation by human neutrophils. PLoS One *10*, e0124082.

**Gabriel, C., McMaster, W.R., Girard, D., and Descoteaux, A.** (2010). Leishmania donovani promastigotes evade the antimicrobial activity of neutrophil extracellular traps. J Immunol *185*, 4319-27.

**Gaipl, U.S., Beyer, T.D., Heyder, P., Kuenkele, S., Bottcher, A.*, et al.*** (2004). Cooperation between C1q and DNase I in the clearance of necrotic cell-derived chromatin. Arthritis Rheum *50*, 640-9.

**Gales, A., Conduche, A., Bernad, J., Lefevre, L., Olagnier, D.*, et al.*** (2010). PPARgamma controls Dectin-1 expression required for host antifungal defense against Candida albicans. PLoS Pathog *6*, e1000714.

**Gantner, B.N., Simmons, R.M., and Underhill, D.M.** (2005). Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments. EMBO J *24*, 1277-86.

**Garcia-Romo, G.S., Caielli, S., Vega, B., Connolly, J., Allantaz, F.*, et al.*** (2011). Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med *3*, 73ra20.

**Gazendam, R.P., van Hamme, J.L., Tool, A.T., van Houdt, M., Verkuijlen, P.J.*, et al.*** (2014). Two independent killing mechanisms of Candida albicans by human neutrophils: evidence from innate immunity defects. Blood *124*, 590-7.

**Geng, S., Matsushima, H., Okamoto, T., Yao, Y., Lu, R.*, et al.*** (2013). Emergence, origin, and function of neutrophil-dendritic cell hybrids in experimentally induced inflammatory lesions in mice. Blood *121*, 1690-700.

**Gerber, C.E., Bruchelt, G., Falk, U.B., Kimpfler, A., Hauschild, O.*, et al.*** (2001). Reconstitution of bactericidal activity in chronic granulomatous disease cells by glucose-oxidase-containing liposomes. Blood *98*, 3097-105.

**Gillenius, E., and Urban, C.F.** (2015). The adhesive protein invasin of Yersinia pseudotuberculosis induces neutrophil extracellular traps via beta1 integrins. Microbes Infect *17*, 327-36.

**Gillum, A.M., Tsay, E.Y., and Kirsch, D.R.** (1984). Isolation of the Candida albicans gene for orotidine-5'-phosphate decarboxylase by complementation of S. cerevisiae ura3 and E. coli pyrF mutations. Mol Gen Genet *198*, 179-82.

**Gladiator, A., Wangler, N., Trautwein-Weidner, K., and LeibundGut-Landmann, S.** (2013). Cutting edge: IL-17-secreting innate lymphoid cells are essential for host defense against fungal infection. J Immunol *190*, 521-5.

**Glocker, E.O., Hennigs, A., Nabavi, M., Schaffer, A.A., Woellner, C.*, et al.*** (2009). A homozygous CARD9 mutation in a family with susceptibility to fungal infections. N Engl J Med *361*, 1727-35.

**Gombart, A.F., Shiohara, M., Kwok, S.H., Agematsu, K., Komiyama, A.*, et al.*** (2001). Neutrophil-specific granule deficiency: homozygous recessive inheritance of a frameshift mutation in the gene encoding transcription factor CCAAT/enhancer binding protein--epsilon. Blood *97*, 2561-7.

**Gonias, S.L., Pasqua, J.J., Greenberg, C., and Pizzo, S.V.** (1985). Precipitation of fibrinogen, fibrinogen degradation products and fibrin monomer by histone H3. Thromb Res *39*, 97-116.

**Goodridge, H.S., Reyes, C.N., Becker, C.A., Katsumoto, T.R., Ma, J.*, et al.*** (2011). Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. Nature *472*, 471-5.

**Görgens, A., Radtke, S., Mollmann, M., Cross, M., Durig, J.*, et al.*** (2013). Revision of the human hematopoietic tree: granulocyte subtypes derive from distinct hematopoietic lineages. Cell Rep *3*, 1539-52.

**Gould, T.J., Lysov, Z., and Liaw, P.C.** (2015). Extracellular DNA and histones: double-edged swords in immunothrombosis. J Thromb Haemost *13 Suppl 1*, S82-91.

**Gould, T.J., Vu, T.T., Swystun, L.L., Dwivedi, D.J., Mai, S.H.*, et al.*** (2014). Neutrophil extracellular traps promote thrombin generation through platelet-dependent and platelet-independent mechanisms. Arterioscler Thromb Vasc Biol *34*, 1977-84.

**Gow, N.A., van de Veerdonk, F.L., Brown, A.J., and Netea, M.G.** (2012). Candida albicans morphogenesis and host defence: discriminating invasion from colonization. Nat Rev Microbiol *10*, 112-22.

**Granick, J.L., Falahee, P.C., Dahmubed, D., Borjesson, D.L., Miller, L.S.*, et al.*** (2013). Staphylococcus aureus recognition by hematopoietic stem and progenitor cells via TLR2/MyD88/PGE2 stimulates granulopoiesis in wounds. Blood *122*, 1770-8.

**Gregory, A.D., and Houghton, A.M.** (2011). Tumor-associated neutrophils: new targets for cancer therapy. Cancer Res *71*, 2411-6.

**Grenda, D.S., Murakami, M., Ghatak, J., Xia, J., Boxer, L.A.*, et al.*** (2007). Mutations of the ELA2 gene found in patients with severe congenital neutropenia induce the unfolded protein response and cellular apoptosis. Blood *110*, 4179-87.

**Griffin, F.M., Jr., Griffin, J.A., Leider, J.E., and Silverstein, S.C.** (1975). Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. J Exp Med *142*, 1263-82.

**Grinberg, N., Elazar, S., Rosenshine, I., and Shpigel, N.Y.** (2008). Beta-hydroxybutyrate abrogates formation of bovine neutrophil extracellular traps and bactericidal activity against mammary pathogenic Escherichia coli. Infect Immun *76*, 2802-7.

**Gringhuis, S.I., Kaptein, T.M., Wevers, B.A., Theelen, B., van der Vlist, M.*, et al.*** (2012). Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1beta via a noncanonical caspase-8 inflammasome. Nat Immunol *13*, 246-54.

**Gross, O., Gewies, A., Finger, K., Schafer, M., Sparwasser, T.*, et al.*** (2006). Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. Nature *442*, 651-6.

**Gross, O., Poeck, H., Bscheider, M., Dostert, C., Hannesschlager, N.*, et al.*** (2009). Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. Nature *459*, 433-6.

**Gudlaugsson, O., Gillespie, S., Lee, K., Vande Berg, J., Hu, J.*, et al.*** (2003). Attributable mortality of nosocomial candidemia, revisited. Clin Infect Dis *37*, 1172-7.

**Guimaraes-Costa, A.B., Nascimento, M.T., Froment, G.S., Soares, R.P., Morgado, F.N.*, et al.*** (2009). Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. Proc Natl Acad Sci U S A *106*, 6748-53.

**Gunderson, C.W., and Seifert, H.S.** (2015). Neisseria gonorrhoeae elicits extracellular traps in primary neutrophil culture while suppressing the oxidative burst. MBio *6*.

**Guo, H., and Wu, X.** (2009). Innate responses of corneal epithelial cells against Aspergillus fumigatus challenge. FEMS Immunol Med Microbiol *56*, 88-93.

**Gupta, A.K., Giaglis, S., Hasler, P., and Hahn, S.** (2014). Efficient neutrophil extracellular trap induction requires mobilization of both intracellular and extracellular calcium pools and is modulated by cyclosporine A. PLoS One *9*, e97088.

**Gupta, A.K., Joshi, M.B., Philippova, M., Erne, P., Hasler, P.*, et al.*** (2010). Activated endothelial cells induce neutrophil extracellular traps and are susceptible to NETosis-mediated cell death. FEBS Lett *584*, 3193-7.

**Hagiwara, T., Nakashima, K., Hirano, H., Senshu, T., and Yamada, M.** (2002). Deimination of arginine residues in nucleophosmin/B23 and histones in HL-60 granulocytes. Biochem Biophys Res Commun *290*, 979-83.

**Hakkim, A., Fuchs, T.A., Martinez, N.E., Hess, S., Prinz, H.*, et al.*** (2011). Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. Nature Chemical Biology *7*, 75-77.

**Hakkim, A., Furnrohr, B.G., Amann, K., Laube, B., Abed, U.A.*, et al.*** (2010). Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci U S A *107*, 9813-8.

**Hall, R.A., Bates, S., Lenardon, M.D., Maccallum, D.M., Wagener, J.*, et al.*** (2013). The Mnn2 mannosyltransferase family modulates mannoprotein fibril length, immune recognition and virulence of Candida albicans. PLoS Pathog *9*, e1003276.

**Hall, R.A., De Sordi, L., Maccallum, D.M., Topal, H., Eaton, R.*, et al.*** (2010). CO(2) acts as a signalling molecule in populations of the fungal pathogen Candida albicans. PLoS Pathog *6*, e1001193.

**Halle, A., Hornung, V., Petzold, G.C., Stewart, C.R., Monks, B.G.*, et al.*** (2008). The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat Immunol *9*, 857-65.

**Hamada, F., Aoki, M., Akiyama, T., and Toyoshima, K.** (1993). Association of immunoglobulin G Fc receptor II with Src-like protein-tyrosine kinase Fgr in neutrophils. Proc Natl Acad Sci U S A *90*, 6305-9.

**Hardison, S.E., and Brown, G.D.** (2012). C-type lectin receptors orchestrate antifungal immunity. Nat Immunol *13*, 817-22.

**Hardison, S.E., Herrera, G., Young, M.L., Hole, C.R., Wozniak, K.L.*, et al.*** (2012). Protective immunity against pulmonary cryptococcosis is associated with STAT1-mediated classical macrophage activation. J Immunol *189*, 4060-8.

**Harris, E.S., Weyrich, A.S., and Zimmerman, G.A.** (2013). Lessons from rare maladies: leukocyte adhesion deficiency syndromes. Curr Opin Hematol *20*, 16-25.

**Hayashi, F., Means, T.K., and Luster, A.D.** (2003). Toll-like receptors stimulate human neutrophil function. Blood *102*, 2660-9.

**Hemmers, S., Teijaro, J.R., Arandjelovic, S., and Mowen, K.A.** (2011). PAD4-mediated neutrophil extracellular trap formation is not required for immunity against influenza infection. PLoS One *6*, e22043.

**Hernandez, P.A., Gorlin, R.J., Lukens, J.N., Taniuchi, S., Bohinjec, J.*, et al.*** (2003). Mutations in the chemokine receptor gene CXCR4 are associated with WHIM syndrome, a combined immunodeficiency disease. Nat Genet *34*, 70-4.

**Hernandez-Santos, N., and Gaffen, S.L.** (2012). Th17 cells in immunity to Candida albicans. Cell Host Microbe *11*, 425-35.

**Hernanz-Falcón, P., Joffre, O., Williams, D.L., and Reis e Sousa, C.** (2009). Internalization of Dectin-1 terminates induction of inflammatory responses. Eur J Immunol *39*, 507-13.

**Herre, J., Marshall, A.S., Caron, E., Edwards, A.D., Williams, D.L.*, et al.*** (2004). Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. Blood *104*, 4038-45.

**Heyworth, P.G., Curnutte, J.T., Nauseef, W.M., Volpp, B.D., Pearson, D.W.*, et al.*** (1991). Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox and p67-phox requires interaction between p47-phox and cytochrome b558. J Clin Invest *87*, 352-6.

**Hirai, H., Kamio, N., Huang, G., Matsusue, A., Ogino, S.*, et al.*** (2013). Cyclic AMP responsive element binding proteins are involved in 'emergency' granulopoiesis through the upregulation of CCAAT/enhancer binding protein beta. PLoS One *8*, e54862.

**Hirai, H., Zhang, P., Dayaram, T., Hetherington, C.J., Mizuno, S.*, et al.*** (2006). C/EBPbeta is required for 'emergency' granulopoiesis. Nat Immunol *7*, 732-9.

**Hise, A.G., Tomalka, J., Ganesan, S., Patel, K., Hall, B.A.*, et al.*** (2009). An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen Candida albicans. Cell Host Microbe *5*, 487-97.

**Ho-Tin-Noe, B., Carbo, C., Demers, M., Cifuni, S.M., Goerge, T.*, et al.*** (2009). Innate immune cells induce hemorrhage in tumors during thrombocytopenia. Am J Pathol *175*, 1699-708.

**Hobson, R.P., Munro, C.A., Bates, S., MacCallum, D.M., Cutler, J.E.*, et al.*** (2004). Loss of cell wall mannosylphosphate in Candida albicans does not influence macrophage recognition. J Biol Chem *279*, 39628-35.

**Hock, H., Hamblen, M.J., Rooke, H.M., Schindler, J.W., Saleque, S.*, et al.*** (2004). Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. Nature *431*, 1002-7.

**Hock, H., Hamblen, M.J., Rooke, H.M., Traver, D., Bronson, R.T.*, et al.*** (2003). Intrinsic requirement for zinc finger transcription factor Gfi-1 in neutrophil differentiation. Immunity *18*, 109-20.

**Holowka, D., Sil, D., Torigoe, C., and Baird, B.** (2007). Insights into immunoglobulin E receptor signaling from structurally defined ligands. Immunol Rev *217*, 269-79.

**Hong, W., Juneau, R.A., Pang, B., and Swords, W.E.** (2009). Survival of bacterial biofilms within neutrophil extracellular traps promotes nontypeable Haemophilus influenzae persistence in the chinchilla model for otitis media. J Innate Immun *1*, 215-24.

**Hopman, R.K., and DiPersio, J.F.** (2014). Advances in stem cell mobilization. Blood Rev *28*, 31-40.

**Hoppe, A.D., and Swanson, J.A.** (2004). Cdc42, Rac1, and Rac2 display distinct patterns of activation during phagocytosis. Mol Biol Cell *15*, 3509-19.

**Horman, S.R., Velu, C.S., Chaubey, A., Bourdeau, T., Zhu, J.*, et al.*** (2009). Gfi1 integrates progenitor versus granulocytic transcriptional programming. Blood *113*, 5466-75.

**Hornung, V., Bauernfeind, F., Halle, A., Samstad, E.O., Kono, H.*, et al.*** (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol *9*, 847-56.

**Houghton, A.M., Rzymkiewicz, D.M., Ji, H., Gregory, A.D., Egea, E.E.*, et al.*** (2010). Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. Nat Med *16*, 219-23.

**Hu, Y., Kiely, J.M., Szente, B.E., Rosenzweig, A., and Gimbrone, M.A., Jr.** (2000). E-selectin-dependent signaling via the mitogen-activated protein kinase pathway in vascular endothelial cells. J Immunol *165*, 2142-8.

**Huang, A.J., Manning, J.E., Bandak, T.M., Ratau, M.C., Hanser, K.R.*, et al.*** (1993). Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells. J Cell Biol *120*, 1371-80.

**Huard, B., McKee, T., Bosshard, C., Durual, S., Matthes, T.*, et al.*** (2008). APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa. J Clin Invest *118*, 2887-95.

**Hube, B.** (2009). Fungal adaptation to the host environment. Curr Opin Microbiol *12*, 347-9.

**Hueber, A.J., Asquith, D.L., Miller, A.M., Reilly, J., Kerr, S.*, et al.*** (2010). Mast cells express IL-17A in rheumatoid arthritis synovium. J Immunol *184*, 3336-40.

**Huppler, A.R., Conti, H.R., Hernandez-Santos, N., Darville, T., Biswas, P.S.*, et al.*** (2014). Role of neutrophils in IL-17-dependent immunity to mucosal candidiasis. J Immunol *192*, 1745-52.

**Hurrell, B.P., Schuster, S., Grun, E., Coutaz, M., Williams, R.A.*, et al.*** (2015). Rapid Sequestration of Leishmania mexicana by Neutrophils Contributes to the Development of Chronic Lesion. PLoS Pathog *11*, e1004929.

**Hyun, Y.M., Sumagin, R., Sarangi, P.P., Lomakina, E., Overstreet, M.G.*, et al.*** (2012). Uropod elongation is a common final step in leukocyte extravasation through inflamed vessels. J Exp Med *209*, 1349-62.

**Ishikawa, T., Itoh, F., Yoshida, S., Saijo, S., Matsuzawa, T.*, et al.*** (2013). Identification of Distinct Ligands for the C-type Lectin Receptors Mincle and Dectin-2 in the Pathogenic Fungus Malassezia. Cell Host Microbe *13*, 477-88.

**Islam, A., Li, S.S., Oykhman, P., Timm-McCann, M., Huston, S.M.*, et al.*** (2013). An acidic microenvironment increases NK cell killing of Cryptococcus neoformans and Cryptococcus gattii by enhancing perforin degranulation. PLoS Pathog *9*, e1003439.

**Itagaki, K., Kaczmarek, E., Lee, Y.T., Tang, I.T., Isal, B.*, et al.*** (2015). Mitochondrial DNA released by trauma induces neutrophil extracellular traps. PLoS One *10*, e0120549.

**Iwasaki, H., Somoza, C., Shigematsu, H., Duprez, E.A., Iwasaki-Arai, J.*, et al.*** (2005). Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. Blood *106*, 1590-600.

**Jaeger, B.N., Donadieu, J., Cognet, C., Bernat, C., Ordonez-Rueda, D.*, et al.*** (2012). Neutrophil depletion impairs natural killer cell maturation, function, and homeostasis. J Exp Med *209*, 565-80.

**Jahn, B., Stuben, A., and Bhakdi, S.** (1996). Colorimetric susceptibility testing for Aspergillus fumigatus: comparison of menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide and Alamar blue tests. J Clin Microbiol *34*, 2039-41.

**Jankowski, A., Scott, C.C., and Grinstein, S.** (2002). Determinants of the phagosomal pH in neutrophils. J Biol Chem *277*, 6059-66.

**Jayaprakash, K., Demirel, I., Khalaf, H., and Bengtsson, T.** (2015). The role of phagocytosis, oxidative burst and neutrophil extracellular traps in the interaction between neutrophils and the periodontal pathogen Porphyromonas gingivalis. Mol Oral Microbiol *30*, 361-75.

**Jenne, C.N., Wong, C.H., Zemp, F.J., McDonald, B., Rahman, M.M.*, et al.*** (2013). Neutrophils recruited to sites of infection protect from virus challenge by releasing neutrophil extracellular traps. Cell Host Microbe *13*, 169-80.

**Jéru, I., Duquesnoy, P., Fernandes-Alnemri, T., Cochet, E., Yu, J.W.*, et al.*** (2008). Mutations in NALP12 cause hereditary periodic fever syndromes. Proc Natl Acad Sci U S A *105*, 1614-9.

**Jiao, J., Dragomir, A.C., Kocabayoglu, P., Rahman, A.H., Chow, A.*, et al.*** (2014). Central role of conventional dendritic cells in regulation of bone marrow release and survival of neutrophils. J Immunol *192*, 3374-82.

**Johnson, M.B., and Criss, A.K.** (2013). Neisseria gonorrhoeae phagosomes delay fusion with primary granules to enhance bacterial survival inside human neutrophils. Cell Microbiol *15*, 1323-40.

**Johnson, S.A., Pleiman, C.M., Pao, L., Schneringer, J., Hippen, K.*, et al.*** (1995). Phosphorylated immunoreceptor signaling motifs (ITAMs) exhibit unique abilities to bind and activate Lyn and Syk tyrosine kinases. J Immunol *155*, 4596-603.

**Joly, S., Ma, N., Sadler, J.J., Soll, D.R., Cassel, S.L.*, et al.*** (2009). Cutting edge: Candida albicans hyphae formation triggers activation of the Nlrp3 inflammasome. J Immunol *183*, 3578-81.

**Joshi, M.B., Lad, A., Bharath Prasad, A.S., Balakrishnan, A., Ramachandra, L.*, et al.*** (2013). High glucose modulates IL-6 mediated immune homeostasis through impeding neutrophil extracellular trap formation. FEBS Letters *587*, 2241-46.

**Juneau, R.A., Pang, B., Armbruster, C.E., Murrah, K.A., Perez, A.C.*, et al.*** (2015a). Peroxiredoxin-glutaredoxin and catalase promote resistance of nontypeable Haemophilus influenzae 86-028NP to oxidants and survival within neutrophil extracellular traps. Infect Immun *83*, 239-46.

**Juneau, R.A., Pang, B., Weimer, K.E., Armbruster, C.E., and Swords, W.E.** (2011). Nontypeable Haemophilus influenzae initiates formation of neutrophil extracellular traps. Infect Immun *79*, 431-8.

**Juneau, R.A., Stevens, J.S., Apicella, M.A., and Criss, A.K.** (2015b). A thermonuclease of Neisseria gonorrhoeae enhances bacterial escape from killing by neutrophil extracellular traps. J Infect Dis *212*, 316-24.

**Kameoka, Y., Persad, A.S., and Suzuki, K.** (2004). Genomic variations in myeloperoxidase gene in the Japanese population. Japanese Journal of Infectious Diseases *57*, S12-S13.

**Kamoshida, G., Kikuchi-Ueda, T., Tansho-Nagakawa, S., Nakano, R., Nakano, A., *et al.*** (2015). Acinetobacter baumannii escape from neutrophil extracellular traps (NETs). J Infect Chemother *21*, 43-9.

**Kanther, M., Tomkovich, S., Xiaolun, S., Grosser, M.R., Koo, J., *et al.*** (2014). Commensal microbiota stimulate systemic neutrophil migration through induction of serum amyloid A. Cell Microbiol *16*, 1053-67.

**Kaplan, M.J., and Radic, M.** (2012). Neutrophil extracellular traps: double-edged swords of innate immunity. J Immunol *189*, 2689-95.

**Karsunky, H., Zeng, H., Schmidt, T., Zevnik, B., Kluge, R., *et al.*** (2002). Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. Nat Genet *30*, 295-300.

**Kashem, S.W., Igyarto, B.Z., Gerami-Nejad, M., Kumamoto, Y., Mohammed, J., *et al.*** (2015a). Candida albicans morphology and dendritic cell subsets determine T helper cell differentiation. Immunity *42*, 356-66.

**Kashem, S.W., Riedl, M.S., Yao, C., Honda, C.N., Vulchanova, L., *et al.*** (2015b). Nociceptive Sensory Fibers Drive Interleukin-23 Production from CD301b+ Dermal Dendritic Cells and Drive Protective Cutaneous Immunity. Immunity *43*, 515-26.

**Kasmapour, B., Gronow, A., Bleck, C.K., Hong, W., and Gutierrez, M.G.** (2012). Size-dependent mechanism of cargo sorting during lysosome-phagosome fusion is controlled by Rab34. Proc Natl Acad Sci U S A *109*, 20485-90.

**Kawakami, M., Tsutsumi, H., Kumakawa, T., Abe, H., Hirai, M., *et al.*** (1990). Levels of serum granulocyte colony-stimulating factor in patients with infections. Blood *76*, 1962-4.

**Kelly, M.N., Zheng, M., Ruan, S., Kolls, J., D'Souza, A., *et al.*** (2013). Memory CD4+ T cells are required for optimal NK cell effector functions against the opportunistic fungal pathogen Pneumocystis murina. J Immunol *190*, 285-95.

**Kerscher, B., Willment, J.A., and Brown, G.D.** (2013). The Dectin-2 family of C-type lectin-like receptors: an update. Int Immunol *25*, 271-7.

**Khandpur, R., Carmona-Rivera, C., Vivekanandan-Giri, A., Gizinski, A., Yalavarthi, S., *et al.*** (2013). NETs Are a Source of Citrullinated Autoantigens and Stimulate Inflammatory Responses in Rheumatoid Arthritis. Sci Transl Med *5*, 178ra40.

**Kjeldsen, L., Bjerrum, O.W., Askaa, J., and Borregaard, N.** (1992). Subcellular localization and release of human neutrophil gelatinase, confirming the existence of separate gelatinase-containing granules. Biochemical Journal *287*, 603-10.

**Klausen, P., Bjerregaard, M.D., Borregaard, N., and Cowland, J.B.** (2004). End-stage differentiation of neutrophil granulocytes in vivo is accompanied by up-regulation of p27kip1 and down-regulation of CDK2, CDK4, and CDK6. J Leukoc Biol *75*, 569-78.

**Klebanoff, S.J.** (2005). Myeloperoxidase: friend and foe. Journal of Leukocyte Biology *77*, 598-625.

**Knight, J.S., Carmona-Rivera, C., and Kaplan, M.J.** (2012). Proteins derived from neutrophil extracellular traps may serve as self-antigens and mediate organ damage in autoimmune diseases. Front Immunol *3*, 380.

**Kolaczkowska, E., and Kubes, P.** (2013). Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol *13*, 159-75.

**Kollner, I., Sodeik, B., Schreek, S., Heyn, H., von Neuhoff, N.*, et al.*** (2006). Mutations in neutrophil elastase causing congenital neutropenia lead to cytoplasmic protein accumulation and induction of the unfolded protein response. Blood *108*, 493-500.

**Korkmaz, B., Horwitz, M.S., Jenne, D.E., and Gauthier, F.** (2010). Neutrophil Elastase, Proteinase 3, and Cathepsin G as Therapeutic Targets in Human Diseases. Pharmacological Reviews *62*, 726-59.

**Kreisel, D., Nava, R.G., Li, W., Zinselmeyer, B.H., Wang, B.*, et al.*** (2010). In vivo two-photon imaging reveals monocyte-dependent neutrophil extravasation during pulmonary inflammation. Proc Natl Acad Sci U S A *107*, 18073-8.

**Kruger, P., Saffarzadeh, M., Weber, A.N., Rieber, N., Radsak, M.*, et al.*** (2015). Neutrophils: Between host defence, immune modulation, and tissue injury. PLoS Pathog *11*, e1004651.

**Kumar, H., Kumagai, Y., Tsuchida, T., Koenig, P.A., Satoh, T.*, et al.*** (2009). Involvement of the NLRP3 inflammasome in innate and humoral adaptive immune responses to fungal beta-glucan. J Immunol *183*, 8061-7.

**Kutter, D.** (1998). Prevalence of myeloperoxidase deficiency: population studies using Bayer-Technicon automated hematology. Journal of Molecular Medicine-Jmm *76*, 669-75.

**Kutter, D., Devaquet, P., Vanderstocken, G., Paulus, J.M., Marchal, V.*, et al.*** (2000). Consequences of total and subtotal myeloperoxidase deficiency: risk or benefit ? Acta Haematol *104*, 10-5.

**Laboratories, B.R.** (1986). BRL pUC host: E. coli DH5αTM competent cells. Bethesda Res Lab Focus, 9–12.

**Lam, J.S., Huang, H., and Levitz, S.M.** (2007). Effect of differential N-linked and O-linked mannosylation on recognition of fungal antigens by dendritic cells. PLoS One *2*, e1009.

**Lamkanfi, M., Malireddi, R.K., and Kanneganti, T.D.** (2009). Fungal zymosan and mannan activate the cryopyrin inflammasome. J Biol Chem *284*, 20574-81.

**Lammermann, T.** (2015). In the eye of the neutrophil swarm-navigation signals that bring neutrophils together in inflamed and infected tissues. J Leukoc Biol.

**Lammermann, T., Afonso, P.V., Angermann, B.R., Wang, J.M., Kastenmuller, W.*, et al.*** (2013). Neutrophil swarms require LTB4 and integrins at sites of cell death in vivo. Nature *498*, 371-5.

**Lande, R., Ganguly, D., Facchinetti, V., Frasca, L., Conrad, C.*, et al.*** (2011). Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. Sci Transl Med *3*, 73ra19.

**Langley, R.G., Elewski, B.E., Lebwohl, M., Reich, K., Griffiths, C.E.*, et al.*** (2014). Secukinumab in plaque psoriasis--results of two phase 3 trials. N Engl J Med *371*, 326-38.

**Lapidot, T., and Kollet, O.** (2002). The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m(null) mice. Leukemia *16*, 1992-2003.

**LaRock, C.N., Dohrmann, S., Todd, J., Corriden, R., Olson, J.*, et al.*** (2015). Group A Streptococcal M1 Protein Sequesters Cathelicidin to Evade Innate Immune Killing. Cell Host Microbe *18*, 471-7.

**Larsen, E.C., Ueyama, T., Brannock, P.M., Shirai, Y., Saito, N.*, et al.*** (2002). A role for PKC-epsilon in Fc gammaR-mediated phagocytosis by RAW 264.7 cells. J Cell Biol *159*, 939-44.

**Latz, E., Xiao, T.S., and Stutz, A.** (2013). Activation and regulation of the inflammasomes. Nat Rev Immunol *13*, 397-411.

**Lauth, X., von Kockritz-Blickwede, M., McNamara, C.W., Myskowski, S., Zinkernagel, A.S.*, et al.*** (2009). M1 protein allows Group A streptococcal survival in phagocyte extracellular traps through cathelicidin inhibition. J Innate Immun *1*, 202-14.

**Le Cabec, V., Cowland, J.B., Calafat, J., and Borregaard, N.** (1996). Targeting of proteins to granule subsets is determined by timing and not by sorting: The specific granule protein NGAL is localized to azurophil granules when expressed in HL-60 cells. Proc Natl Acad Sci U S A *93*, 6454-7.

**Lee, W.L., Harrison, R.E., and Grinstein, S.** (2003). Phagocytosis by neutrophils. Microbes Infect *5*, 1299-306.

**Leffler, J., Martin, M., Gullstrand, B., Tyden, H., Lood, C.*, et al.*** (2012). Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. J Immunol *188*, 3522-31.

**LeibundGut-Landmann, S., Gross, O., Robinson, M.J., Osorio, F., Slack, E.C.*, et al.*** (2007). Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nat Immunol *8*, 630-8.

**Leshner, M., Wang, S., Lewis, C., Zheng, H., Chen, X.A.*, et al.*** (2012). PAD4 mediated histone hypercitrullination induces heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures. Front Immunol *3*, 307.

**Leslie, R.D., and Bradford, C.** (2014). Autoimmune diabetes: caught in a NET. Diabetes *63*, 4018-20.

**Levitz, S.M., and Diamond, R.D.** (1985). A rapid colorimetric assay of fungal viability with the tetrazolium salt MTT. J Infect Dis *152*, 938-45.

**Ley, K., Laudanna, C., Cybulsky, M.I., and Nourshargh, S.** (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol *7*, 678-89.

**Ley, K., Smith, E., and Stark, M.A.** (2006). IL-17A-producing neutrophil-regulatory Tn lymphocytes. Immunol Res *34*, 229-42.

**Li, P., Li, M., Lindberg, M.R., Kennett, M.J., Xiong, N.*, et al.*** (2010). PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. J Exp Med *207*, 1853-62.

**Lieschke, G.J., Grail, D., Hodgson, G., Metcalf, D., Stanley, E.*, et al.*** (1994). Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood *84*, 1737-46.

**Lin, A.M., Rubin, C.J., Khandpur, R., Wang, J.Y., Riblett, M.*, et al.*** (2011). Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. J Immunol *187*, 490-500.

**Lionakis, M.S., Swamydas, M., Fischer, B.G., Plantinga, T.S., Johnson, M.D.*, et al.*** (2013). CX3CR1-dependent renal macrophage survival promotes Candida control and host survival. J Clin Invest *123*, 5035-51.

**Lippolis, J.D., Reinhardt, T.A., Goff, J.P., and Horst, R.L.** (2006). Neutrophil extracellular trap formation by bovine neutrophils is not inhibited by milk. Vet Immunol Immunopathol *113*, 248-55.

**Liu, L., Okada, S., Kong, X.F., Kreins, A.Y., Cypowyj, S.*, et al.*** (2011). Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. J Exp Med *208*, 1635-48.

**Liu, X., Ma, B., Malik, A.B., Tang, H., Yang, T.*, et al.*** (2012). Bidirectional regulation of neutrophil migration by mitogen-activated protein kinases. Nat Immunol *13*, 457-64.

**Longhi, L.N., da Silva, R.M., Fornazim, M.C., Spago, M.C., de Oliveira, R.T.*, et al.*** (2012). Phenotypic and functional characterization of NK cells in human immune response against the dimorphic fungus Paracoccidioides brasiliensis. J Immunol *189*, 935-45.

**Loures, F.V., Rohm, M., Lee, C.K., Santos, E., Wang, J.P.*, et al.*** (2015). Recognition of Aspergillus fumigatus hyphae by human plasmacytoid dendritic cells is mediated by dectin-2 and results in formation of extracellular traps. PLoS Pathog *11*, e1004643.

**Lowman, D.W., Greene, R.R., Bearden, D.W., Kruppa, M.D., Pottier, M.*, et al.*** (2014). Novel structural features in Candida albicans hyphal glucan provide a basis for differential innate immune recognition of hyphae versus yeast. J Biol Chem *289*, 3432-43.

**Mahajan, A., Herrmann, M., and Munoz, L.E.** (2016). Clearance Deficiency and Cell Death Pathways: A Model for the Pathogenesis of SLE. Front Immunol *7*, 35.

**Maidan, M.M., De Rop, L., Serneels, J., Exler, S., Rupp, S.*, et al.*** (2005). The G protein-coupled receptor Gpr1 and the Galpha protein Gpa2 act through the cAMP-protein kinase A pathway to induce morphogenesis in Candida albicans. Mol Biol Cell *16*, 1971-86.

**Malachowa, N., Kobayashi, S.D., Freedman, B., Dorward, D.W., and DeLeo, F.R.** (2013). Staphylococcus aureus leukotoxin GH promotes formation of neutrophil extracellular traps. J Immunol *191*, 6022-9.

**Malemud, C.J.** (2006). Matrix metalloproteinases (MMPs) in health and disease: an overview. Front Biosci *11*, 1696-701.

**Mankan, A.K., Dau, T., Jenne, D., and Hornung, V.** (2012). The NLRP3/ASC/Caspase-1 axis regulates IL-1beta processing in neutrophils. Eur J Immunol *42*, 710-5.

**Mantovani, A., Cassatella, M.A., Costantini, C., and Jaillon, S.** (2011). Neutrophils in the activation and regulation of innate and adaptive immunity. Nat Rev Immunol *11*, 519-31.

**Manz, M.G., and Boettcher, S.** (2014). Emergency granulopoiesis. Nat Rev Immunol *14*, 302-14.

**Marakalala, M.J., Vautier, S., Potrykus, J., Walker, L.A., Shepardson, K.M.*, et al.*** (2013). Differential adaptation of Candida albicans in vivo modulates immune recognition by dectin-1. PLoS Pathog *9*, e1003315.

**Marcos, V., Zhou, Z., Yildirim, A.O., Bohla, A., Hector, A.*, et al.*** (2010). CXCR2 mediates NADPH oxidase-independent neutrophil extracellular trap formation in cystic fibrosis airway inflammation. Nat Med *16*, 1018-U114.

**Mariathasan, S., Weiss, D.S., Newton, K., McBride, J., O'Rourke, K.*, et al.*** (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. Nature *440*, 228-32.

**Marr, K.A., Balajee, S.A., Hawn, T.R., Ozinsky, A., Pham, U.*, et al.*** (2003). Differential role of MyD88 in macrophage-mediated responses to opportunistic fungal pathogens. Infect Immun *71*, 5280-6.

**Martin, C., Burdon, P.C., Bridger, G., Gutierrez-Ramos, J.C., Williams, T.J.*, et al.*** (2003). Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. Immunity *19*, 583-93.

**Martinelli, R., Gegg, M., Longbottom, R., Adamson, P., Turowski, P.*, et al.*** (2009). ICAM-1-mediated endothelial nitric oxide synthase activation via calcium and AMP-activated protein kinase is required for transendothelial lymphocyte migration. Mol Biol Cell *20*, 995-1005.

**Martinelli, S., Urosevic, M., Daryadel, A., Oberholzer, P.A., Baumann, C.*, et al.*** (2004). Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. J Biol Chem *279*, 44123-32.

**Martinod, K., Fuchs, T.A., Zitomersky, N.L., Wong, S.L., Demers, M.*, et al.*** (2015). PAD4-deficiency does not affect bacteremia in polymicrobial sepsis and ameliorates endotoxemic shock. Blood *125*, 1948-56.

**Martinon, F., Petrilli, V., Mayor, A., Tardivel, A., and Tschopp, J.** (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature *440*, 237-41.

**Maslowski, K.M., Vieira, A.T., Ng, A., Kranich, J., Sierro, F.*, et al.*** (2009). Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature *461*, 1282-6.

**Matsushima, H., Geng, S., Lu, R., Okamoto, T., Yao, Y.*, et al.*** (2013). Neutrophil differentiation into a unique hybrid population exhibiting dual phenotype and functionality of neutrophils and dendritic cells. Blood *121*, 1677-89.

**May, R.C., Caron, E., Hall, A., and Machesky, L.M.** (2000). Involvement of the Arp2/3 complex in phagocytosis mediated by FcgammaR or CR3. Nat Cell Biol *2*, 246-8.

**McCormick, A., Heesemann, L., Wagener, J., Marcos, V., Hartl, D.*, et al.*** (2010). NETs formed by human neutrophils inhibit growth of the pathogenic mold Aspergillus fumigatus. Microbes Infect *12*, 928-36.

**McDonald, B., Pittman, K., Menezes, G.B., Hirota, S.A., Slaba, I.*, et al.*** (2010). Intravascular danger signals guide neutrophils to sites of sterile inflammation. Science *330*, 362-6.

**McDonald, B., Urrutia, R., Yipp, B.G., Jenne, C.N., and Kubes, P.** (2012). Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. Cell Host Microbe *12*, 324-33.

**McKercher, S.R., Torbett, B.E., Anderson, K.L., Henkel, G.W., Vestal, D.J.*, et al.*** (1996). Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J *15*, 5647-58.

**Meier, A., Kirschning, C.J., Nikolaus, T., Wagner, H., Heesemann, J.*, et al.*** (2003). Toll-like receptor (TLR) 2 and TLR4 are essential for Aspergillus-induced activation of murine macrophages. Cell Microbiol *5*, 561-70.

**Meijering, E., Jacob, M., Sarria, J.C., Steiner, P., Hirling, H.*, et al.*** (2004). Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. Cytometry A *58*, 167-76.

**Mejia, S.P., Cano, L.E., Lopez, J.A., Hernandez, O., and Gonzalez, A.** (2015). Human neutrophils produce extracellular traps against Paracoccidioides brasiliensis. Microbiology *161*, 1008-17.

**Mencacci, A., Bacci, A., Cenci, E., Montagnoli, C., Fiorucci, S.*, et al.*** (2000). Interleukin 18 restores defective Th1 immunity to Candida albicans in caspase 1-deficient mice. Infect Immun *68*, 5126-31.

**Menegazzi, R., Decleva, E., and Dri, P.** (2012). Killing by neutrophil extracellular traps: fact or folklore? Blood *119*, 1214-16.

**Menegazzo, L., Ciciliot, S., Poncina, N., Mazzucato, M., Persano, M.*, et al.*** (2015). NETosis is induced by high glucose and associated with type 2 diabetes. Acta Diabetol *52*, 497-503.

**Metzler, K.D., Fuchs, T.A., Nauseef, W.M., Reumaux, D., Roesler, J.*, et al.*** (2011). Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. Blood *117*, 953-9.

**Metzler, Kathleen D., Goosmann, C., Lubojemska, A., Zychlinsky, A., and Papayannopoulos, V.** (2014). A Myeloperoxidase-Containing Complex Regulates Neutrophil Elastase Release and Actin Dynamics during NETosis. Cell Reports.

**Millan, J., Hewlett, L., Glyn, M., Toomre, D., Clark, P.*, et al.*** (2006). Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains. Nat Cell Biol *8*, 113-23.

**Mitroulis, I., Kambas, K., Chrysanthopoulou, A., Skendros, P., Apostolidou, E.*, et al.*** (2011). Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. PLoS One *6*, e29318.

**Mócsai, A., Abram, C.L., Jakus, Z., Hu, Y., Lanier, L.L.*, et al.*** (2006). Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. Nat Immunol *7*, 1326-33.

**Mócsai, A., Ruland, J., and Tybulewicz, V.L.** (2010). The SYK tyrosine kinase: a crucial player in diverse biological functions. Nat Rev Immunol *10*, 387-402.

**Mollinedo, F., Calafat, J., Janssen, H., Martin-Martin, B., Canchado, J.*, et al.*** (2006). Combinatorial SNARE complexes modulate the secretion of cytoplasmic granules in human neutrophils. J Immunol *177*, 2831-41.

**Mora-Montes, H.M., Bates, S., Netea, M.G., Castillo, L., Brand, A.*, et al.*** (2010). A multifunctional mannosyltransferase family in Candida albicans determines cell wall mannan structure and host-fungus interactions. J Biol Chem *285*, 12087-95.

**Morosetti, R., Park, D.J., Chumakov, A.M., Grillier, I., Shiohara, M.*, et al.*** (1997). A novel, myeloid transcription factor, C/EBP epsilon, is upregulated during granulocytic, but not monocytic, differentiation. Blood *90*, 2591-600.

**Mosser, D.M., and Edwards, J.P.** (2008). Exploring the full spectrum of macrophage activation. Nat Rev Immunol *8*, 958-69.

**Moutsopoulos, N.M., Konkel, J., Sarmadi, M., Eskan, M.A., Wild, T.*, et al.*** (2014). Defective neutrophil recruitment in leukocyte adhesion deficiency type I disease causes local IL-17-driven inflammatory bone loss. Sci Transl Med *6*, 229ra40.

**Moyes, D.L., Murciano, C., Runglall, M., Islam, A., Thavaraj, S.*, et al.*** (2011). Candida albicans yeast and hyphae are discriminated by MAPK signaling in vaginal epithelial cells. PLoS One *6*, e26580.

**Moyes, D.L., Runglall, M., Murciano, C., Shen, C., Nayar, D.*, et al.*** (2010). A biphasic innate immune MAPK response discriminates between the yeast and hyphal forms of Candida albicans in epithelial cells. Cell Host Microbe *8*, 225-35.

**Muller, S., and Radic, M.** (2015). Citrullinated Autoantigens: From Diagnostic Markers to Pathogenetic Mechanisms. Clin Rev Allergy Immunol *49*, 232-9.

**Muller, U., Stenzel, W., Kohler, G., Werner, C., Polte, T.*, et al.*** (2007). IL-13 induces disease-promoting type 2 cytokines, alternatively activated macrophages and allergic inflammation during pulmonary infection of mice with Cryptococcus neoformans. J Immunol *179*, 5367-77.

**Muller, W.A.** (2011). Mechanisms of leukocyte transendothelial migration. Annu Rev Pathol *6*, 323-44.

**Munafo, D.B., Johnson, J.L., Brzezinska, A.A., Ellis, B.A., Wood, M.R.*, et al.*** (2009). DNase I inhibits a late phase of reactive oxygen species production in neutrophils. J Innate Immun *1*, 527-42.

**Munoz-Caro, T., Lendner, M., Daugschies, A., Hermosilla, C., and Taubert, A.** (2015a). NADPH oxidase, MPO, NE, ERK1/2, p38 MAPK and Ca2+ influx are essential for Cryptosporidium parvum-induced NET formation. Dev Comp Immunol *52*, 245-54.

**Munoz-Caro, T., Mena Huertas, S.J., Conejeros, I., Alarcon, P., Hidalgo, M.A.*, et al.*** (2015b). Eimeria bovis-triggered neutrophil extracellular trap formation is CD11b-, ERK 1/2-, p38 MAP kinase- and SOCE-dependent. Vet Res *46*, 23.

**Munro, C.A., Schofield, D.A., Gooday, G.W., and Gow, N.A.** (1998). Regulation of chitin synthesis during dimorphic growth of Candida albicans. Microbiology *144 ( Pt 2)*, 391-401.

**Murphy, K., Travers, P., Walport, M., and Janeway, C.A., Jr.** (2012). Janeway's immunobiology 8th ed. edn (United States: New York : Garland Science, c2012.).

**Nagaraj, S., Youn, J.I., and Gabrilovich, D.I.** (2013). Reciprocal relationship between myeloid-derived suppressor cells and T cells. J Immunol *191*, 17-23.

**Nagelkerke, S.Q., aan de Kerk, D.J., Jansen, M.H., van den Berg, T.K., and Kuijpers, T.W.** (2014). Failure to detect functional neutrophil B helper cells in the human spleen. PLoS One *9*, e88377.

**Nahrendorf, M., and Swirski, F.K.** (2015). Immunology. Neutrophil-macrophage communication in inflammation and atherosclerosis. Science *349*, 237-8.

**Nakamura, H., Fang, J., Mizukami, T., Nunoi, H., and Maeda, H.** (2012). PEGylated D-amino acid oxidase restores bactericidal activity of neutrophils in chronic granulomatous disease via hypochlorite. Exp Biol Med (Maywood) *237*, 703-8.

**Nantel, A., Dignard, D., Bachewich, C., Harcus, D., Marcil, A.*, et al.*** (2002). Transcription profiling of Candida albicans cells undergoing the yeast-to-hyphal transition. Mol Biol Cell *13*, 3452-65.

**Narasaraju, T., Yang, E., Samy, R.P., Ng, H.H., Poh, W.P.*, et al.*** (2011). Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. Am J Pathol *179*, 199-210.

**Nauseef, W.M.** (2007). How human neutrophils kill and degrade microbes: an integrated view. Immunological Reviews *219*, 88-102.

**Nauseef, W.M., Volpp, B.D., McCormick, S., Leidal, K.G., and Clark, R.A.** (1991). Assembly of the neutrophil respiratory burst oxidase. Protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. J Biol Chem *266*, 5911-7.

**Neeli, I., Khan, S.N., and Radic, M.** (2008). Histone deimination as a response to inflammatory stimuli in neutrophils. Journal of Immunology *180*, 1895-902.

**Neeli, I., and Radic, M.** (2013). Opposition between PKC isoforms regulates histone deimination and neutrophil extracellular chromatin release. Front Immunol *4*, 38.

**Nerlov, C., and Graf, T.** (1998). PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. Genes Dev *12*, 2403-12.

**Netea, M.G., Brown, G.D., Kullberg, B.J., and Gow, N.A.** (2008). An integrated model of the recognition of Candida albicans by the innate immune system. Nat Rev Microbiol *6*, 67-78.

**Netea, M.G., Gijzen, K., Coolen, N., Verschueren, I., Figdor, C.*, et al.*** (2004). Human dendritic cells are less potent at killing Candida albicans than both monocytes and macrophages. Microbes Infect *6*, 985-9.

**Netea, M.G., Gow, N.A., Munro, C.A., Bates, S., Collins, C.*, et al.*** (2006). Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. J Clin Invest *116*, 1642-50.

**Netea, M.G., Joosten, L.A., van der Meer, J.W., Kullberg, B.J., and van de Veerdonk, F.L.** (2015a). Immune defence against Candida fungal infections. Nat Rev Immunol *15*, 630-42.

**Netea, M.G., Nold-Petry, C.A., Nold, M.F., Joosten, L.A., Opitz, B.*, et al.*** (2009). Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. Blood *113*, 2324-35.

**Netea, M.G., van de Veerdonk, F.L., van der Meer, J.W., Dinarello, C.A., and Joosten, L.A.** (2015b). Inflammasome-independent regulation of IL-1-family cytokines. Annu Rev Immunol *33*, 49-77.

**Netea, M.G., Van Der Graaf, C.A., Vonk, A.G., Verschueren, I., Van Der Meer, J.W., *et al.*** (2002). The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. J Infect Dis *185*, 1483-9.

**Netea, M.G., Warris, A., Van der Meer, J.W., Fenton, M.J., Verver-Janssen, T.J., *et al.*** (2003). Aspergillus fumigatus evades immune recognition during germination through loss of toll-like receptor-4-mediated signal transduction. J Infect Dis *188*, 320-6.

**Ng, T.H., Chang, S.H., Wu, M.H., and Wang, H.C.** (2013). Shrimp hemocytes release extracellular traps that kill bacteria. Dev Comp Immunol *41*, 644-51.

**Ngo, L.Y., Kasahara, S., Kumasaka, D.K., Knoblaugh, S.E., Jhingran, A., *et al.*** (2014). Inflammatory monocytes mediate early and organ-specific innate defense during systemic candidiasis. J Infect Dis *209*, 109-19.

**Nimmerjahn, F., Fau - Ravetch, J.V., and Ravetch, J.V.** (2008). Fcgamma receptors as regulators of immune responses. *2008*.

**Nordenfelt, P., and Tapper, H.** (2011). Phagosome dynamics during phagocytosis by neutrophils. J Leukoc Biol *90*, 271-84.

**Nordenfelt, P., Winberg, M.E., Lonnbro, P., Rasmusson, B., and Tapper, H.** (2009). Different requirements for early and late phases of azurophilic granule-phagosome fusion. Traffic *10*, 1881-93.

**Nourshargh, S., and Alon, R.** (2014). Leukocyte migration into inflamed tissues. Immunity *41*, 694-707.

**Nourshargh, S., Hordijk, P.L., and Sixt, M.** (2010). Breaching multiple barriers: leukocyte motility through venular walls and the interstitium. Nat Rev Mol Cell Biol *11*, 366-78.

**O'Mahony, D.S., Pham, U., Iyer, R., Hawn, T.R., and Liles, W.C.** (2008). Differential Constitutive and Cytokine-Modulated Expression of Human Toll-like Receptors in Primary Neutrophils, Monocytes, and Macrophages. International Journal of Medical Sciences *5*, 1-8.

**Odobasic, D., Kitching, A.R., Yang, Y., O'Sullivan, K.M., Muljadi, R.C., *et al.*** (2013). Neutrophil myeloperoxidase regulates T-cell-driven tissue inflammation in mice by inhibiting dendritic cell function. Blood *121*, 4195-204.

**Ohkubo, T., Tsuda, M., Tamura, M., and Yamamura, M.** (1990). Impaired superoxide production in peripheral blood neutrophils of germ-free rats. Scand J Immunol *32*, 727-9.

**Orkin, S.H., and Zon, L.I.** (2008). SnapShot: hematopoiesis. Cell *132*, 712.

**Osorio, F., and Reis e Sousa, C.** (2011). Myeloid C-type lectin receptors in pathogen recognition and host defense. Immunity *34*, 651-64.

**Palmer, L.J., Cooper, P.R., Ling, M.R., Wright, H.J., Huissoon, A., *et al.*** (2012). Hypochlorous acid regulates neutrophil extracellular trap release in humans. Clin Exp Immunol *167*, 261-8.

**Panopoulos, A.D., and Watowich, S.S.** (2008). Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and 'emergency' hematopoiesis. Cytokine *42*, 277-88.

**Papayannopoulos, V., Metzler, K.D., Hakkim, A., and Zychlinsky, A.** (2010). Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol *191*, 677-91.

**Papayannopoulos, V., Staab, D., and Zychlinsky, A.** (2011). Neutrophil elastase enhances sputum solubilization in cystic fibrosis patients receiving DNase therapy. PLoS One *6*, e28526.

**Pappas, P.G.** (2010). Opportunistic fungi: a view to the future. Am J Med Sci *340*, 253-7.

**Parker, H., Albrett, A.M., Kettle, A.J., and Winterbourn, C.C.** (2012a). Myeloperoxidase associated with neutrophil extracellular traps is active and mediates bacterial killing in the presence of hydrogen peroxide. Journal of Leukocyte Biology *91*, 369-76.

**Parker, H., Dragunow, M., Hampton, M.B., Kettle, A.J., and Winterbourn, C.C.** (2012b). Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. J Leukoc Biol.

**Parker, H., and Winterbourn, C.C.** (2012). Reactive oxidants and myeloperoxidase and their involvement in neutrophil extracellular traps. Front Immunol *3*, 424.

**Parker, L.C., Whyte, M.K., Dower, S.K., and Sabroe, I.** (2005). The expression and roles of Toll-like receptors in the biology of the human neutrophil. J Leukoc Biol *77*, 886-92.

**Pelletier, M., Maggi, L., Micheletti, A., Lazzeri, E., Tamassia, N.*, et al.*** (2010). Evidence for a cross-talk between human neutrophils and Th17 cells. Blood *115*, 335-43.

**Perlroth, J., Choi, B., and Spellberg, B.** (2007). Nosocomial fungal infections: epidemiology, diagnosis, and treatment. Med Mycol *45*, 321-46.

**Person, R.E., Li, F.Q., Duan, Z., Benson, K.F., Wechsler, J.*, et al.*** (2003). Mutations in proto-oncogene GFI1 cause human neutropenia and target ELA2. Nat Genet *34*, 308-12.

**Pietrella, D., Pandey, N., Gabrielli, E., Pericolini, E., Perito, S.*, et al.*** (2013). Secreted aspartic proteases of Candida albicans activate the NLRP3 inflammasome. Eur J Immunol *43*, 679-92.

**Pillay, J., den Braber, I., Vrisekoop, N., Kwast, L.M., de Boer, R.J.*, et al.*** (2010). In vivo labeling with (H2O)-H-2 reveals a human neutrophil lifespan of 5.4 days. Blood *116*, 625-27.

**Pillay, J., Kamp, V.M., van Hoffen, E., Visser, T., Tak, T.*, et al.*** (2012). A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. J Clin Invest *122*, 327-36.

**Pilsczek, F.H., Salina, D., Poon, K.K., Fahey, C., Yipp, B.G.*, et al.*** (2010). A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. J Immunol *185*, 7413-25.

**Pober, J.S., and Sessa, W.C.** (2007). Evolving functions of endothelial cells in inflammation. Nat Rev Immunol *7*, 803-15.

**Pober, J.S., and Tellides, G.** (2012). Participation of blood vessel cells in human adaptive immune responses. Trends Immunol *33*, 49-57.

**Prince, L.R., Whyte, M.K., Sabroe, I., and Parker, L.C.** (2011). The role of TLRs in neutrophil activation. Curr Opin Pharmacol *11*, 397-403.

**Proebstl, D., Voisin, M.B., Woodfin, A., Whiteford, J., D'Acquisto, F.*, et al.*** (2012). Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls in vivo. J Exp Med *209*, 1219-34.

**Puel, A., Cypowyj, S., Bustamante, J., Wright, J.F., Liu, L.*, et al.*** (2011). Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. Science *332*, 65-8.

**Puga, I., Cols, M., Barra, C.M., He, B., Cassis, L.*, et al.*** (2012). B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. Nat Immunol *13*, 170-80.

**Qian, Q., Jutila, M.A., Van Rooijen, N., and Cutler, J.E.** (1994). Elimination of mouse splenic macrophages correlates with increased susceptibility to experimental disseminated candidiasis. J Immunol *152*, 5000-8.

**Quintin, J., Saeed, S., Martens, J.H., Giamarellos-Bourboulis, E.J., Ifrim, D.C.*, et al.*** (2012). Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host Microbe *12*, 223-32.

**Quintin, J., Voigt, J., van der Voort, R., Jacobsen, I.D., Verschueren, I.*, et al.*** (2014). Differential role of NK cells against Candida albicans infection in immunocompetent or immunocompromised mice. Eur J Immunol *44*, 2405-14.

**Radomska, H.S., Huettner, C.S., Zhang, P., Cheng, T., Scadden, D.T.*, et al.*** (1998). CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. Mol Cell Biol *18*, 4301-14.

**Ramirez-Ortiz, Z.G., Lee, C.K., Wang, J.P., Boon, L., Specht, C.A.*, et al.*** (2011). A nonredundant role for plasmacytoid dendritic cells in host defense against the human fungal pathogen Aspergillus fumigatus. Cell Host Microbe *9*, 415-24.

**Ramirez-Ortiz, Z.G., Specht, C.A., Wang, J.P., Lee, C.K., Bartholomeu, D.C.*, et al.*** (2008). Toll-like receptor 9-dependent immune activation by unmethylated CpG motifs in Aspergillus fumigatus DNA. Infect Immun *76*, 2123-9.

**Ramos-Kichik, V., Mondragon-Flores, R., Mondragon-Castelan, M., Gonzalez-Pozos, S., Muniz-Hernandez, S.*, et al.*** (2009). Neutrophil extracellular traps are induced by Mycobacterium tuberculosis. Tuberculosis (Edinb) *89*, 29-37.

**Ratjen, F.** (2008). Recent advances in cystic fibrosis. Paediatr Respir Rev *9*, 144-8.

**Ratliff, T.L., Ritchey, J.K., Brandhorst, J., and Hanna, M.G., Jr.** (1994). Time-dependent aggregation of reconstituted BCG vaccine. J Urol *152*, 2147-50.

**Reddy, V.A., Iwama, A., Iotzova, G., Schulz, M., Elsasser, A.*, et al.*** (2002). Granulocyte inducer C/EBPalpha inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions. Blood *100*, 483-90.

**Renesto, P., and Chignard, M.** (1993). Enhancement of cathepsin G-induced platelet activation by leukocyte elastase: consequence for the neutrophil-mediated platelet activation. Blood *82*, 139-44.

**Ribeiro-Gomes, F.L., Peters, N.C., Debrabant, A., and Sacks, D.L.** (2012). Efficient capture of infected neutrophils by dendritic cells in the skin inhibits the early anti-leishmania response. PLoS Pathog *8*, e1002536.

**Riyapa, D., Buddhisa, S., Korbsrisate, S., Cuccui, J., Wren, B.W.*, et al.*** (2012). Neutrophil extracellular traps exhibit antibacterial activity against burkholderia pseudomallei and are influenced by bacterial and host factors. Infect Immun *80*, 3921-9.

**Robert, R., Nail, S., Marot-Leblond, A., Cottin, J., Miegeville, M.*, et al.*** (2000). Adherence of platelets to Candida species in vivo. Infect Immun *68*, 570-6.

**Roberts, A.W.** (2005). G-CSF: a key regulator of neutrophil production, but that's not all! Growth Factors *23*, 33-41.

**Roberts, P.J., Pizzey, A.R., Khwaja, A., Carver, J.E., Mire-Sluis, A.R.*, et al.*** (1993). The effects of interleukin-8 on neutrophil fMetLeuPhe receptors, CD11b expression and metabolic activity, in comparison and combination with other cytokines. Br J Haematol *84*, 586-94.

**Robinson, M.J., Osorio, F., Rosas, M., Freitas, R.P., Schweighoffer, E.*, et al.*** (2009). Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. J Exp Med *206*, 2037-51.

**Rohm, M., Grimm, M.J., D'Auria, A.C., Almyroudis, N.G., Segal, B.H.*, et al.*** (2014). NADPH oxidase promotes neutrophil extracellular trap formation in pulmonary aspergillosis. Infect Immun *82*, 1766-77.

**Romani, L.** (2004). Immunity to fungal infections. Nat Rev Immunol *4*, 1-23.

**Romani, L.** (2011). Immunity to fungal infections. Nat Rev Immunol *11*, 275-88.

**Romani, L., Bistoni, F., and Puccetti, P.** (2002). Fungi, dendritic cells and receptors: a host perspective of fungal virulence. Trends Microbiol *10*, 508-14.

**Romani, L., Bistoni, F., and Puccetti, P.** (2003). Adaptation of Candida albicans to the host environment: the role of morphogenesis in virulence and survival in mammalian hosts. Curr Opin Microbiol *6*, 338-43.

**Romani, L., Mencacci, A., Cenci, E., Spaccapelo, R., Schiaffella, E.*, et al.*** (1993). Natural killer cells do not play a dominant role in CD4+ subset differentiation in Candida albicans-infected mice. Infect Immun *61*, 3769-74.

**Romani, L., and Puccetti, P.** (2006). Protective tolerance to fungi: the role of IL-10 and tryptophan catabolism. Trends Microbiol *14*, 183-9.

**Rørvig, S., Ostergaard, O., Heegaard, N.H., and Borregaard, N.** (2013). Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors. J Leukoc Biol *94*, 711-21.

**Saeed, S., Quintin, J., Kerstens, H.H., Rao, N.A., Aghajanirefah, A.*, et al.*** (2014). Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. Science *345*, 1251086.

**Saijo, S., Fujikado, N., Furuta, T., Chung, S.H., Kotaki, H.*, et al.*** (2007). Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. Nat Immunol *8*, 39-46.

**Saijo, S., Ikeda, S., Yamabe, K., Kakuta, S., Ishigame, H.*, et al.*** (2010). Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against Candida albicans. Immunity *32*, 681-91.

**Saitoh, T., Komano, J., Saitoh, Y., Misawa, T., Takahama, M.*, et al.*** (2012). Neutrophil Extracellular Traps Mediate a Host Defense Response to Human Immunodeficiency Virus-1. Cell Host Microbe *12*, 109-16.

**Sandor, N., Kristof, K., Parej, K., Pap, D., Erdei, A.*, et al.*** (2013). CR3 is the dominant phagocytotic complement receptor on human dendritic cells. Immunobiology *218*, 652-63.

**Scapini, P., Carletto, A., Nardelli, B., Calzetti, F., Roschke, V.*, et al.*** (2005). Proinflammatory mediators elicit secretion of the intracellular B-lymphocyte stimulator pool (BLyS) that is stored in activated neutrophils: implications for inflammatory diseases. Blood *105*, 830-7.

**Scapini, P., and Cassatella, M.A.** (2014). Social networking of human neutrophils within the immune system. Blood *124*, 710-9.

**Schaffner, A., Davis, C.E., Schaffner, T., Markert, M., Douglas, H.*, et al.*** (1986). In vitro susceptibility of fungi to killing by neutrophil granulocytes discriminates between primary pathogenicity and opportunism. J Clin Invest *78*, 511-24.

**Scharrig, E., Carestia, A., Ferrer, M.F., Cedola, M., Pretre, G.*, et al.*** (2015). Neutrophil Extracellular Traps are Involved in the Innate Immune Response to Infection with Leptospira. PLoS Negl Trop Dis *9*, e0003927.

**Schauer, C., Janko, C., Munoz, L.E., Zhao, Y., Kienhofer, D.*, et al.*** (2014). Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. Nat Med *20*, 511-7.

**Scheiermann, C., Kunisaki, Y., Lucas, D., Chow, A., Jang, J.E.*, et al.*** (2012). Adrenergic nerves govern circadian leukocyte recruitment to tissues. Immunity *37*, 290-301.

**Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M.*, et al.*** (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods *9*, 676-82.

**Schmidt, S., Tramsen, L., Perkhofer, S., Lass-Florl, C., Hanisch, M.*, et al.*** (2013a). Rhizopus oryzae hyphae are damaged by human natural killer (NK) cells, but suppress NK cell mediated immunity. Immunobiology *218*, 939-44.

**Schmidt, S., Zimmermann, S.Y., Tramsen, L., Koehl, U., and Lehrnbecher, T.** (2013b). Natural killer cells and antifungal host response. Clin Vaccine Immunol *20*, 452-8.

**Schorn, C., Janko, C., Krenn, V., Zhao, Y., Munoz, L.E.*, et al.*** (2012). Bonding the foe - NETting neutrophils immobilize the pro-inflammatory monosodium urate crystals. Front Immunol *3*, 376.

**Schwarzenberger, P., Huang, W., Ye, P., Oliver, P., Manuel, M.*, et al.*** (2000). Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis. J Immunol *164*, 4783-9.

**Segal, A.W., Dorling, J., and Coade, S.** (1980). Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies. J Cell Biol *85*, 42-59.

**Segal, B.H., Leto, T.L., Gallin, J.I., Malech, H.L., and Holland, S.M.** (2000). Genetic, biochemical, and clinical features of chronic granulomatous disease. Medicine (Baltimore) *79*, 170-200.

**Selig, C., and Nothdurft, W.** (1995). Cytokines and progenitor cells of granulocytopoiesis in peripheral blood of patients with bacterial infections. Infect Immun *63*, 104-9.

**Semerad, C.L., Liu, F., Gregory, A.D., Stumpf, K., and Link, D.C.** (2002). G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood. Immunity *17*, 413-23.

**Sengelov, H., Follin, P., Kjeldsen, L., Lollike, K., Dahlgren, C.*, et al.*** (1995). Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J Immunol *154*, 4157-65.

**Sengelov, H., Kjeldsen, L., and Borregaard, N.** (1993). Control of exocytosis in early neutrophil activation. Journal of Immunology *150*, 1535-43.

**Shapiro, R.S., Uppuluri, P., Zaas, A.K., Collins, C., Senn, H.*, et al.*** (2009). Hsp90 orchestrates temperature-dependent Candida albicans morphogenesis via Ras1-PKA signaling. Curr Biol *19*, 621-9.

**Shulman, Z., Shinder, V., Klein, E., Grabovsky, V., Yeger, O.*, et al.*** (2009). Lymphocyte crawling and transendothelial migration require chemokine triggering of high-affinity LFA-1 integrin. Immunity *30*, 384-96.

**Si-Tahar, M., Pidard, D., Balloy, V., Moniatte, M., Kieffer, N.*, et al.*** (1997). Human neutrophil elastase proteolytically activates the platelet integrin alphaIIbbeta3 through cleavage of the carboxyl terminus of the alphaIIb subunit heavy chain. Involvement in the potentiation of platelet aggregation. J Biol Chem *272*, 11636-47.

**Smeekens, S.P., van de Veerdonk, F.L., Kullberg, B.J., and Netea, M.G.** (2013). Genetic susceptibility to Candida infections. EMBO Mol Med *5*, 805-13.

**Sohn, K., Urban, C., Brunner, H., and Rupp, S.** (2003). EFG1 is a major regulator of cell wall dynamics in Candida albicans as revealed by DNA microarrays. Mol Microbiol *47*, 89-102.

**Sorensen, O.E., Clemmensen, S.N., Dahl, S.L., Ostergaard, O., Heegaard, N.H.*, et al.*** (2014). Papillon-Lefevre syndrome patient reveals species-dependent requirements for neutrophil defenses. J Clin Invest *124*, 4539-48.

**Spellberg, B., Ibrahim, A.S., Lin, L., Avanesian, V., Fu, Y.*, et al.*** (2008). Antibody titer threshold predicts anti-candidal vaccine efficacy even though the mechanism of protection is induction of cell-mediated immunity. J Infect Dis *197*, 967-71.

**Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J.P.*, et al.*** (2013). Innate lymphoid cells--a proposal for uniform nomenclature. Nat Rev Immunol *13*, 145-9.

**Springer, D.J., Ren, P., Raina, R., Dong, Y., Behr, M.J.*, et al.*** (2010). Extracellular fibrils of pathogenic yeast Cryptococcus gattii are important for ecological niche, murine virulence and human neutrophil interactions. PLoS One *5*, e10978.

**Standish, A.J., and Weiser, J.N.** (2009). Human neutrophils kill Streptococcus pneumoniae via serine proteases. J Immunol *183*, 2602-9.

**Stark, K., Eckart, A., Haidari, S., Tirniceriu, A., Lorenz, M.*, et al.*** (2013). Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and 'instruct' them with pattern-recognition and motility programs. Nat Immunol *14*, 41-51.

**Stark, M.A., Huo, Y., Burcin, T.L., Morris, M.A., Olson, T.S.*, et al.*** (2005). Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. Immunity *22*, 285-94.

**Stasia, M.J., and Li, X.J.** (2008). Genetics and immunopathology of chronic granulomatous disease. Semin Immunopathol *30*, 209-35.

**Strasser, D., Neumann, K., Bergmann, H., Marakalala, M.J., Guler, R.*, et al.*** (2012). Syk kinase-coupled C-type lectin receptors engage protein kinase C-sigma to elicit Card9 adaptor-mediated innate immunity. Immunity *36*, 32-42.

**Sudbery, P., Gow, N., and Berman, J.** (2004). The distinct morphogenic states of Candida albicans. Trends Microbiol *12*, 317-24.

**Sumby, P., Barbian, K.D., Gardner, D.J., Whitney, A.R., Welty, D.M.*, et al.*** (2005). Extracellular deoxyribonuclease made by group A Streptococcus assists pathogenesis by enhancing evasion of the innate immune response. Proc Natl Acad Sci U S A *102*, 1679-84.

**Sun, W.K., Lu, X., Li, X., Sun, Q.Y., Su, X.*, et al.*** (2012). Dectin-1 is inducible and plays a crucial role in Aspergillus-induced innate immune responses in human bronchial epithelial cells. Eur J Clin Microbiol Infect Dis *31*, 2755-64.

**Sundd, P., Gutierrez, E., Koltsova, E.K., Kuwano, Y., Fukuda, S.*, et al.*** (2012). 'Slings' enable neutrophil rolling at high shear. Nature *488*, 399-403.

**Szymczak, W.A., and Deepe, G.S., Jr.** (2009). The CCL7-CCL2-CCR2 axis regulates IL-4 production in lungs and fungal immunity. J Immunol *183*, 1964-74.

**Taborda, C.P., and Casadevall, A.** (2002). CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are involved in complement-independent antibody-mediated phagocytosis of Cryptococcus neoformans. Immunity *16*, 791-802.

**Tada, H., Nemoto, E., Shimauchi, H., Watanabe, T., Mikami, T.*, et al.*** (2002). Saccharomyces cerevisiae- and Candida albicans-derived mannan induced production of tumor necrosis factor alpha by human monocytes in a CD14- and Toll-like receptor 4-dependent manner. Microbiol Immunol *46*, 503-12.

**Tak, T., Tesselaar, K., Pillay, J., Borghans, J.A., and Koenderman, L.** (2013). What's your age again? Determination of human neutrophil half-lives revisited. J Leukoc Biol *94*, 595-601.

**Tamassia, N., Le Moigne, V., Calzetti, F., Donini, M., Gasperini, S.*, et al.*** (2007). The MyD88-independent pathway is not mobilized in human neutrophils stimulated via TLR4. J Immunol *178*, 7344-56.

**Tamassia, N., Le Moigne, V., Rossato, M., Donini, M., McCartney, S.*, et al.*** (2008). Activation of an immunoregulatory and antiviral gene expression program in poly(I:C)-transfected human neutrophils. J Immunol *181*, 6563-73.

**Tanaka, H., Ishikawa, K., Nishino, M., Shimazu, T., and Yoshioka, T.** (1996). Changes in granulocyte colony-stimulating factor concentration in patients with trauma and sepsis. J Trauma *40*, 718-25; discussion 25-6.

**Tapper, H., Furuya, W., and Grinstein, S.** (2002). Localized exocytosis of primary (lysosomal) granules during phagocytosis: role of Ca2+-dependent tyrosine phosphorylation and microtubules. J Immunol *168*, 5287-96.

**Taylor, P.R., Tsoni, S.V., Willment, J.A., Dennehy, K.M., Rosas, M.*, et al.*** (2007). Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat Immunol *8*, 31-8.

**Thammavongsa, V., Missiakas, D.M., and Schneewind, O.** (2013). Staphylococcus aureus degrades neutrophil extracellular traps to promote immune cell death. Science *342*, 863-6.

**Theilgaard-Monch, K., Jacobsen, L.C., Borup, R., Rasmussen, T., Bjerregaard, M.D.*, et al.*** (2005). The transcriptional program of terminal granulocytic differentiation. Blood *105*, 1785-96.

**Thomas, C.J., and Schroder, K.** (2013). Pattern recognition receptor function in neutrophils. Trends Immunol *34*, 317-28.

**Tomalka, J., Azodi, E., Narra, H.P., Patel, K., O'Neill, S.*, et al.*** (2015). beta-Defensin 1 plays a role in acute mucosal defense against Candida albicans. J Immunol *194*, 1788-95.

**Touret, N., Paroutis, P., Terebiznik, M., Harrison, R.E., Trombetta, S.*, et al.*** (2005). Quantitative and dynamic assessment of the contribution of the ER to phagosome formation. Cell *123*, 157-70.

**Tsai, P.W., Yang, C.Y., Chang, H.T., and Lan, C.Y.** (2011). Human antimicrobial peptide LL-37 inhibits adhesion of Candida albicans by interacting with yeast cell-wall carbohydrates. PLoS One *6*, e17755.

**Tsokos, G.C.** (2011). Systemic lupus erythematosus. N Engl J Med *365*, 2110-21.

**Urban, C.F., Ermert, D., Schmid, M., Abu-Abed, U., Goosmann, C.*, et al.*** (2009). Neutrophil Extracellular Traps Contain Calprotectin, a Cytosolic Protein Complex Involved in Host Defense against <italic>Candida albicans</italic>. PLoS Pathog *5*, e1000639.

**Urban, C.F., Reichard, U., Brinkmann, V., and Zychlinsky, A.** (2006). Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. Cell Microbiol *8*, 668-76.

**Uzun, O., Ascioglu, S., Anaissie, E.J., and Rex, J.H.** (2001). Risk factors and predictors of outcome in patients with cancer and breakthrough candidemia. Clin Infect Dis *32*, 1713-7.

**van Bruggen, R., Anthony, E., Fernandez-Borja, M., and Roos, D.** (2004). Continuous translocation of Rac2 and the NADPH oxidase component p67(phox) during phagocytosis. J Biol Chem *279*, 9097-102.

**van Bruggen, R., Drewniak, A., Jansen, M., van Houdt, M., Roos, D.*, et al.*** (2009). Complement receptor 3, not Dectin-1, is the major receptor on human neutrophils for beta-glucan-bearing particles. Mol Immunol *47*, 575-81.

**van Bruggen, R., Drewniak, A., Tool, A.T., Jansen, M., van Houdt, M.*, et al.*** (2010). Toll-like receptor responses in IRAK-4-deficient neutrophils. J Innate Immun *2*, 280-7.

**van de Veerdonk, F.L., Kullberg, B.J., van der Meer, J.W., Gow, N.A., and Netea, M.G.** (2008). Host-microbe interactions: innate pattern recognition of fungal pathogens. Curr Opin Microbiol *11*, 305-12.

**Van der Graaf, C.A., Netea, M.G., Morre, S.A., Den Heijer, M., Verweij, P.E.*, et al.*** (2006). Toll-like receptor 4 Asp299Gly/Thr399Ile polymorphisms are a risk factor for Candida bloodstream infection. Eur Cytokine Netw *17*, 29-34.

**van der Meer, J.W., Joosten, L.A., Riksen, N., and Netea, M.G.** (2015). Trained immunity: A smart way to enhance innate immune defence. Mol Immunol *68*, 40-4.

**VanderVen, B.C., Yates, R.M., and Russell, D.G.** (2009). Intraphagosomal measurement of the magnitude and duration of the oxidative burst. Traffic *10*, 372-8.

**Velu, C.S., Baktula, A.M., and Grimes, H.L.** (2009). Gfi1 regulates miR-21 and miR-196b to control myelopoiesis. Blood *113*, 4720-8.

**Vestweber, D.** (2012). Relevance of endothelial junctions in leukocyte extravasation and vascular permeability. Ann N Y Acad Sci *1257*, 184-92.

**Vignais, P.V.** (2002). The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. Cell Mol Life Sci *59*, 1428-59.

**Vijayan, D., Radford, K.J., Beckhouse, A.G., Ashman, R.B., and Wells, C.A.** (2012). Mincle polarizes human monocyte and neutrophil responses to Candida albicans. Immunol Cell Biol *90*, 889-95.

**Villanueva, E., Yalavarthi, S., Berthier, C.C., Hodgin, J.B., Khandpur, R.*, et al.*** (2011). Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. J Immunol *187*, 538-52.

**Viriyakosol, S., Jimenez Mdel, P., Gurney, M.A., Ashbaugh, M.E., and Fierer, J.** (2013). Dectin-1 is required for resistance to coccidioidomycosis in mice. MBio *4*, e00597-12.

**Vogelmeier, C., and Döring, G.** (1996). Neutrophil proteinases and rhDNase therapy in cystic fibrosis. European Respiratory Journal *9*, 2193-95.

**Voisin, M.B., and Nourshargh, S.** (2013). Neutrophil transmigration: emergence of an adhesive cascade within venular walls. J Innate Immun *5*, 336-47.

**Voisin, M.B., Probstl, D., and Nourshargh, S.** (2010). Venular basement membranes ubiquitously express matrix protein low-expression regions: characterization in multiple tissues and remodeling during inflammation. Am J Pathol *176*, 482-95.

**von Bernuth, H., Picard, C., Jin, Z., Pankla, R., Xiao, H.*, et al.*** (2008). Pyogenic bacterial infections in humans with MyD88 deficiency. Science *321*, 691-6.

**von Bruhl, M.L., Stark, K., Steinhart, A., Chandraratne, S., Konrad, I.*, et al.*** (2012). Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med *209*, 819-35.

**von Vietinghoff, S., and Ley, K.** (2009). IL-17A controls IL-17F production and maintains blood neutrophil counts in mice. J Immunol *183*, 865-73.

**Vong, L., Lorentz, R.J., Assa, A., Glogauer, M., and Sherman, P.M.** (2014). Probiotic Lactobacillus rhamnosus inhibits the formation of neutrophil extracellular traps. J Immunol *192*, 1870-7.

**Vong, L., Pinnell, L.J., Maattanen, P., Yeung, C.W., Lurz, E.*, et al.*** (2015). Selective enrichment of commensal gut bacteria protects against Citrobacter rodentium-induced colitis. Am J Physiol Gastrointest Liver Physiol *309*, G181-92.

**Wagener, J., Malireddi, R.K., Lenardon, M.D., Koberle, M., Vautier, S.*, et al.*** (2014). Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation. PLoS Pathog *10*, e1004050.

**Wagener, J., Schneider, J.J., Baxmann, S., Kalbacher, H., Borelli, C.*, et al.*** (2013). A peptide derived from the highly conserved protein GAPDH is involved in tissue protection by different antifungal strategies and epithelial immunomodulation. J Invest Dermatol *133*, 144-53.

**Walcheck, B., Moore, K.L., McEver, R.P., and Kishimoto, T.K.** (1996). Neutrophil-neutrophil interactions under hydrodynamic shear stress involve L-selectin and PSGL-1. A mechanism that amplifies initial leukocyte accumulation of P-selectin in vitro. J Clin Invest *98*, 1081-7.

**Wang, A.V., Scholl, P.R., and Geha, R.S.** (1994). Physical and functional association of the high affinity immunoglobulin G receptor (Fc gamma RI) with the kinases Hck and Lyn. J Exp Med *180*, 1165-70.

**Wang, J.P., Bowen, G.N., Padden, C., Cerny, A., Finberg, R.W.*, et al.*** (2008). Toll-like receptor-mediated activation of neutrophils by influenza A virus. Blood *112*, 2028-34.

**Wang, Y., Xiao, Y., Zhong, L., Ye, D., Zhang, J.*, et al.*** (2014). Increased neutrophil elastase and proteinase 3 and augmented NETosis are closely associated with beta-cell autoimmunity in patients with type 1 diabetes. Diabetes *63*, 4239-48.

**Wang, Y.M., Li, M., Stadler, S., Correll, S., Li, P.X.*, et al.*** (2009). Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. Journal of Cell Biology *184*, 205-13.

**Ward, C.M., Tetaz, T.J., Andrews, R.K., and Berndt, M.C.** (1997). Binding of the von Willebrand factor A1 domain to histone. Thromb Res *86*, 469-77.

**Wardini, A.B., Guimaraes-Costa, A.B., Nascimento, M.T., Nadaes, N.R., Danelli, M.G.*, et al.*** (2010). Characterization of neutrophil extracellular traps in cats naturally infected with feline leukemia virus. J Gen Virol *91*, 259-64.

**Warnatsch, A., Ioannou, M., Wang, Q., and Papayannopoulos, V.** (2015). Inflammation. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. Science *349*, 316-20.

**Wartha, F., Beiter, K., Albiger, B., Fernebro, J., Zychlinsky, A.*, et al.*** (2007). Capsule and D-alanylated lipoteichoic acids protect Streptococcus pneumoniae against neutrophil extracellular traps. Cell Microbiol *9*, 1162-71.

**Weindl, G., Naglik, J.R., Kaesler, S., Biedermann, T., Hube, B.*, et al.*** (2007). Human epithelial cells establish direct antifungal defense through TLR4-mediated signaling. J Clin Invest *117*, 3664-72.

**Weindl, G., Wagener, J., and Schaller, M.** (2010). Epithelial cells and innate antifungal defense. J Dent Res *89*, 666-75.

**Weinrauch, Y., Drujan, D., Shapiro, S.D., Weiss, J., and Zychlinsky, A.** (2002). Neutrophil elastase targets virulence factors of enterobacteria. Nature *417*, 91-94.

**Wells, C.A., Salvage-Jones, J.A., Li, X., Hitchens, K., Butcher, S.*, et al.*** (2008). The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to Candida albicans. J Immunol *180*, 7404-13.

**Werner, J.L., Gessner, M.A., Lilly, L.M., Nelson, M.P., Metz, A.E.*, et al.*** (2011). Neutrophils produce interleukin 17A (IL-17A) in a dectin-1- and IL-23-dependent manner during invasive fungal infection. Infect Immun *79*, 3966-77.

**Werner, J.L., Metz, A.E., Horn, D., Schoeb, T.R., Hewitt, M.M.*, et al.*** (2009). Requisite role for the dectin-1 beta-glucan receptor in pulmonary defense against Aspergillus fumigatus. J Immunol *182*, 4938-46.

**Wessel, F., Winderlich, M., Holm, M., Frye, M., Rivera-Galdos, R.*, et al.*** (2014). Leukocyte extravasation and vascular permeability are each controlled in vivo by different tyrosine residues of VE-cadherin. Nat Immunol *15*, 223-30.

**Whitney, P.G., Bar, E., Osorio, F., Rogers, N.C., Schraml, B.U.*, et al.*** (2014). Syk signaling in dendritic cells orchestrates innate resistance to systemic fungal infection. PLoS Pathog *10*, e1004276.

**Wientjes, F.B., Hsuan, J.J., Totty, N.F., and Segal, A.W.** (1993). p40phox, a third cytosolic component of the activation complex of the NADPH oxidase to contain src homology 3 domains. Biochem J *296 ( Pt 3)*, 557-61.

**Willment, J.A., Lin, H.H., Reid, D.M., Taylor, P.R., Williams, D.L.*, et al.*** (2003). Dectin-1 expression and function are enhanced on alternatively activated and GM-CSF-treated macrophages and are negatively regulated by IL-10, dexamethasone, and lipopolysaccharide. J Immunol *171*, 4569-73.

**Wilson, K.P., Black, J.A., Thomson, J.A., Kim, E.E., Griffith, J.P.*, et al.*** (1994). Structure and mechanism of interleukin-1 beta converting enzyme. Nature *370*, 270-5.

**Winkler, I.G., Barbier, V., Wadley, R., Zannettino, A.C., Williams, S.*, et al.*** (2010). Positioning of bone marrow hematopoietic and stromal cells relative to blood flow in vivo: serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. Blood *116*, 375-85.

**Wong, S.L., Demers, M., Martinod, K., Gallant, M., Wang, Y.*, et al.*** (2015). Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. Nat Med *21*, 815-19.

**Woodfin, A., Voisin, M.B., Beyrau, M., Colom, B., Caille, D.*, et al.*** (2011). The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. Nat Immunol *12*, 761-9.

**Xu, J., Zhang, X., Pelayo, R., Monestier, M., Ammollo, C.T.*, et al.*** (2009). Extracellular histones are major mediators of death in sepsis. Nat Med *15*, 1318-U117.

**Xu, X.L., Lee, R.T., Fang, H.M., Wang, Y.M., Li, R.*, et al.*** (2008). Bacterial peptidoglycan triggers Candida albicans hyphal growth by directly activating the adenylyl cyclase Cyr1p. Cell Host Microbe *4*, 28-39.

**Yamanaka, R., Barlow, C., Lekstrom-Himes, J., Castilla, L.H., Liu, P.P.*, et al.*** (1997). Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. Proc Natl Acad Sci U S A *94*, 13187-92.

**Yamasaki, S., Matsumoto, M., Takeuchi, O., Matsuzawa, T., Ishikawa, E.*, et al.*** (2009). C-type lectin Mincle is an activating receptor for pathogenic fungus, Malassezia. Proc Natl Acad Sci U S A *106*, 1897-902.

**Yang, C.W., and Unanue, E.R.** (2013). Neutrophils control the magnitude and spread of the immune response in a thromboxane A2-mediated process. J Exp Med *210*, 375-87.

**Yauch, L.E., Lam, J.S., and Levitz, S.M.** (2006). Direct inhibition of T-cell responses by the Cryptococcus capsular polysaccharide glucuronoxylomannan. PLoS Pathog *2*, e120.

**Yeh, T.M., Chang, H.C., Liang, C.C., Wu, J.J., and Liu, M.F.** (2003). Deoxyribonuclease-inhibitory antibodies in systemic lupus erythematosus. J Biomed Sci *10*, 544-51.

**Yipp, B.G., Petri, B., Salina, D., Jenne, C.N., Scott, B.N.*, et al.*** (2012). Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. Nat Med *18*, 1386-93.

**Yoo, D.G., Floyd, M., Winn, M., Moskowitz, S.M., and Rada, B.** (2014). NET formation induced by Pseudomonas aeruginosa cystic fibrosis isolates measured as release of myeloperoxidase-DNA and neutrophil elastase-DNA complexes. Immunol Lett *160*, 186-94.

**Youn, J.I., and Gabrilovich, D.I.** (2010). The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity. Eur J Immunol *40*, 2969-75.

**Yousefi, S., Gold, J.A., Andina, N., Lee, J.J., Kelly, A.M.*, et al.*** (2008). Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. Nat Med *14*, 949-53.

**Yousefi, S., Mihalache, C., Kozlowski, E., Schmid, I., and Simon, H.U.** (2009). Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell Death Differ *16*, 1438-44.

**Zarbock, A., Ley, K., McEver, R.P., and Hidalgo, A.** (2011). Leukocyte ligands for endothelial selectins: specialized glycoconjugates that mediate rolling and signaling under flow. Blood *118*, 6743-51.

**Zarebski, A., Velu, C.S., Baktula, A.M., Bourdeau, T., Horman, S.R.*, et al.*** (2008). Mutations in growth factor independent-1 associated with human neutropenia block murine granulopoiesis through colony stimulating factor-1. Immunity *28*, 370-80.

**Zeilhofer, H.U., and Schorr, W.** (2000). Role of interleukin-8 in neutrophil signaling. Curr Opin Hematol *7*, 178-82.

**Zhang, D., Chen, G., Manwani, D., Mortha, A., Xu, C.*, et al.*** (2015). Neutrophil ageing is regulated by the microbiome. Nature *525*, 528-32.

**Zhang, Y., Wang, F., Tompkins, K.C., McNamara, A., Jain, A.V.*, et al.*** (2009). Robust Th1 and Th17 immunity supports pulmonary clearance but cannot prevent systemic dissemination of highly virulent Cryptococcus neoformans H99. Am J Pathol *175*, 2489-500.

**Zhao, J., Pan, S., Lin, L., Fu, L., Yang, C.*, et al.*** (2015). Streptococcus suis serotype 2 strains can induce the formation of neutrophil extracellular traps and evade trapping. FEMS Microbiol Lett *362*.

**Zheng, X., Wang, Y., and Wang, Y.** (2004). Hgc1, a novel hypha-specific G1 cyclin-related protein regulates Candida albicans hyphal morphogenesis. EMBO J *23*, 1845-56.

**Zhou, Y., Wu, J., Kucik, D.F., White, N.B., Redden, D.T.*, et al.*** (2013). Multiple lupus-associated ITGAM variants alter Mac-1 functions on neutrophils. Arthritis Rheum *65*, 2907-16.